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# Methods for identifying frost injury in immature maize seed

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Methods for identifying frost injury in immature maize seed

by

Mindy L. DeVries

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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## TABLE OF CONTENTS

LIST OF FIGURES	v
LIST OF TABLES	vii
ABSTRACT	viii
CHAPTER 1. GENERAL INTRODUCTION	1
Dissertation Organization	1
Literature Review	1
References	16
CHAPTER 2. DETERMINING THE EXTENT OF FROST DAMAGE IN MAIZE SEED USTING THE TETRAZOLIUM TEST	22
Abstract	22
Introduction	22
Production of Frost-Damaged Seed Corn	24
Description of Vigor Categories	26
Relationships among Tetrazolium, Standard Germination, and Saturated Cold Tests	28
Conclusions	29
Acknowledgements	29
Literature Cited	29
CHAPTER 3. SEED QUALITY TESTS FOR EARLY DETECTION OF FALL FROST DAMAGE IN IMMATURE MAIZE SEED	40
Abstract	40
Introduction	41
Materials and Methods	45

Results and Discussion	48
Conclusions	55
Acknowledgements	57
References	57
 CHAPTER 4. MEASURING EXPRESSION OF mRNA SEGMENTS IN DRY MAIZE SEED	 82
Introduction	82
Materials and Methods	85
Results and Discussion	88
Conclusions	91
Acknowledgements	91
References	92
 CHAPTER 5. GENERAL CONCLUSIONS	 110
 ACKNOWLEDGEMENTS	 112

## LIST OF FIGURES

### Chapter 2

Figure 1. A high vigor seed as indicated by the staining pattern of red spots on a white background.

Figure 2. Critical embryo structures seed in a longitudinally bisected corn kernel.

Figure 3. Medium vigor seeds with beginning signs of damage.

Figure 4. Low vigor seeds with extensive damage to the plumule.

Figure 5. Ungerminable seeds as indicated by the absence of stained tissue in critical embryo tissues.

Figure 6. Typical frost-killed seed where the plumule, meristem, and center of the radicle are unstained, whereas all remaining tissues exhibit little damage.

Figure 7. Positive correlation ( $r=0.86^{***}$ ) between TZ test results and the percentage of normal seedlings in the saturated cold test after 6 months of storage.

### Chapter 3

Figure 1. Artificial frost cycle developed to mimic an autumn severe killing frost in the Midwest.

Figure 2. Standard germination at 0 mo (WG0) and late field emergence.

Figure 3. Standard germination at 1.5 mo (WG1) and late field emergence.

Figure 4. Standard germination at 3 mo (WG3) and late field emergence.

Figure 5. Accelerated aging test at 0 mo (AA0) and late field emergence.

Figure 6. Accelerated aging test at 1.5 mo (AA1) and late field emergence.

Figure 7. Accelerated aging test at 3 mo (AA3) and late field emergence.

Figure 8. Saturated cold test at 0 mo (SC0) and late field emergence.

Figure 9. Saturated cold test at 1.5 mo (SC1) and late field emergence.

Figure 10. Saturated cold test at 3 mo (SC3) and late field emergence.

Figure 11. Soak test at 0 mo (Soak0) and late field emergence.

Figure 12. Soak test at 1.5 mo (Soak1) and late field emergence.

Figure 13. Soak test at 3 mo (Soak3) and late field emergence.

**Chapter 4**

Figure 1. Plate design for Real-Time PCR of block 1.

Figure 2. PCR product from all samples on 3% agarose gel.

Figure 3. FAM and ROX background for Sample 1 with good amplification.

Figure 4. FAM and ROX background for a NTC sample showing FAM breakdown.

Figure 5. Amplification plot for 18S endogenous control gene in Sample A with 10 fold serial dilution (10 to 10<sup>8</sup>).

Figure 6. Standard curve for 18S endogenous control gene in Sample A with 10 fold serial dilution (10 to 10<sup>8</sup>).

Figure 7. Amplification plot for the gene of interest (Genebank accession number BQ740237) in Sample B with 4 fold serial dilution (4 to 4<sup>5</sup>).

Figure 8. Standard curve for the gene of interest (Genebank accession number BQ740237) in Sample B with 4 fold serial dilution (4 to 4<sup>5</sup>).

Figure 9. Amplification plot for all twelve samples including 3 replications of 18S and the gene of interest.

Figure 10. Relative expression of SAM decarboxylase with Sample 6 (Mo17 hybrid, harvested at 300-350 g kg<sup>-1</sup>, control) as the calibrator.

Figure 11. Relative expression of SAM decarboxylase with Sample 12 (B73 hybrid, harvested at 300-350 g kg<sup>-1</sup>) as the calibrator.



## LIST OF TABLES

### Chapter 2

Table 1. General description of the vigor categories based on the staining patterns of critical embryo structures.

Table 2. Pearson's Correlation Coefficients between the TZ test at 0 months of storage and the standard germination test and saturated cold test after 0, 3, and 6 months of storage.

### Chapter 3

Table 1. Seed production environments.

Table 2. Field Emergence Conditions.

Table 3. Analysis of variance (degrees of freedom (df) and *P* values) for standard germination and saturated cold test after 0, 1.5, 3, 4.5 and 6 months in storage.

Table 4. Effect of frost treatment on standard germination test for two females harvest at three moisture contents.

Table 5. Effect of frost treatment on accelerated aging test for two females harvest at three moisture contents.

Table 6. Effect of frost treatment on saturated cold test for two females harvest at three moisture contents.

Table 7. Effect of frost treatment on soak test for two females harvest at three moisture contents.

Table 8. Summary of the simple linear regressions between the seed quality tests (WG, AA, SC, and Soak) at 0 and 1.5 months and early field emergence (FE1).

### Chapter 4

Table 1. Spectrophotometer readings of mRNA quality and quantity.

Table 2. Quantities of cDNA in PCR reactions for determining the standard curve for the 18S endogenous control and the gene of interest.

Table 3.  $\Delta\Delta CT$  values for the gene of interest by female and MC combination.

## ABSTRACT

Fall frost damage is a major threat to maize (*Zea mays* L.) seed production in the central United States. Frost events prior to harvest can cause various physical, mechanical, biochemical, and physiological changes to immature seed corn. These changes can lead to decreased germination and vigor. Early detection of frost damage could reduce the financial loss caused by poor emergence when these seed lots are planted. The central hypotheses of this dissertation are that the severity of a frost event can be quantified shortly after seed has been harvested and dried, and that the magnitude of the damage is associated with seed development and genetic background of the seed. This information can be used to predict field emergence of frosted seed lots. Many different aspects of frost damage have been explored in this project, which provides several methods for identifying frost damage in maize seed. This project advances our understanding of seed physiology as related to frost damage and changes in physiology during seed maturation. Chapter 2 is a practical application of the tetrazolium test for identifying frost damage in seed corn and relating these results to vigor. Chapter 3 discusses the influence of female parent and moisture content at harvest in frost tolerance or injury. Chapter 3 also provides a detailed analysis of seed quality tests and their usefulness in predicting field emergence of frost damaged seed. Chapter 4 establishes the use of RNA extraction and qRT-PCR as a valid method for evaluating gene expression in dry maize seed.

## **CHAPTER 1. GENERAL INTRODUCTION**

### **Dissertation Organization**

This dissertation includes three individual experiments on the effects of frost damage on seed quality. These manuscripts are written in manuscript format for submission to scientific journals appropriate for the content of the paper. Chapter 2 is a practical application of the tetrazolium test for identifying frost damage in seed corn and relating these results to vigor (Crop Management, 2006). Chapter 3 discusses the role of female parent and moisture content at harvest in frost tolerance or injury. Chapter 3 also provides a detailed analysis of seed quality tests and their usefulness in predicting field emergence of frost damaged seed. Chapter 4 establishes the use of RNA extraction and qRT-PCR as a valid method for evaluating gene expression from dry maize seed. Figures, tables, and references are listed at the end of each chapter. Lastly, general conclusions are presented.

### **Literature Review**

The American Seed Trade Association estimates maize seed production in the United States is 454,000 metric tons annually. This seed is planted domestically and is exported worldwide. Producing and maintaining high quality seed for sale is the number one goal of seed companies. To attain this goal, it is important to accurately identify those seed lots which have low vigor, viability and quality.

Early fall frosts are not uncommon in Iowa and most of the Midwest. In this region, frost can significantly hinder seed corn production approximately once every five years (Burris and Knittle, 1985). In general, seed lots that have been frosted before harvest have lower seed quality and, therefore, less commercial value than healthy seed lots. Detection of frost damaged seed lots reduces the financial loss caused by poor emergence.

#### **Low Temperature Stress and Freezing**

Plant response to temperature stress can either be elastic or plastic (Levitt, 1980). If the effects of the stress are reversible and do not require additional

metabolism in order to repair, the strain imposed is considered elastic (Levitt, 1980). However, if the effects of the stress are irreversible or require metabolic energy for repair, the strain is considered plastic (Levitt, 1980). Low temperature stress in plants has several plastic components including chilling stress, cold acclimation, frost tolerance, and freezing injury. Our study is limited to the effects of a frost event likely to induce freezing in seed tissue.

#### Frost Events and the Freezing Process

There are two major types of fall frost events in central Iowa, radiation and convective freezing. Radiation freezing occurs when heat from the plants is radiated to the surrounding environment leading to lower internal temperature in the plant tissue than in the surrounding air (Shaw, 1981). This type of frost event occurs at night when there are clear skies and cool temperatures. However, the air temperature does not need to be below 0°C in order for a radiation freeze to occur. Frost on the leaf surface can serve as an ice nucleation point leading to ice formation inside plant tissue (Pearce and Fuller, 2001). Convective freezing, in contrast, is caused by air temperature dropping below 0°C. Once the air and plant temperature drop below 0°C, the ice front moves from the plant surface into the plant tissue. During a convective freeze, plant and air temperatures are close to each other (Shaw, 1981).

Plant tissues are known to supercool prior to ice formation (Mazur, 1969; Hartwigsen, 1999). On the average, corn has the ability to supercool to temperatures of -2.5° C (Fick, 1989; Hartwigsen, 1999). Freezing within plant tissues can occur both in the intracellular (Kiessellbach and Ratcliff, 1920; Burke et al., 1976) and extracellular (Mazur, 1969; Hartwigsen, 1999) regions. Ice nucleation begins in the extracellular region and, as the ice crystals expand, additional water from within the cells migrates into the extracellular region and freezes (Mazur, 1969; Steponkus, 1984). This process of ice formation and cell water migration continues, which is why freezing is similar to dehydration stress. When the temperature is very low or drops very quickly ice crystals actually form in the intracellular region, which causes direct damage to the cells (Rossman, 1949a; Burke et al., 1976).

Ice nucleation is a random event and can occur anywhere within an ear of corn (Fick, 1989). Internal ice nucleation points in plants are hard to locate, but have been reported in leaf blades (Fick, 1989; Pearce and Fuller, 2001). External ice nucleation may be caused by a layer of frost on the surface of plant tissue. This external source of ice can lead to internal nucleation within the plant. Ice nucleation active (INA) bacteria, such as *Pseudomonas syringae* and *Erwinia herbicola*, can induce ice nucleation in plants (Wisniewski et al., 1997; Fick, 1989; Stier et al., 2003). The bacteria prevent supercooling by initiating ice formation. In these studies, the presence of ice nucleators was found to increase frost damage.

The degree of freezing injury in seeds depends on several factors. The minimum temperature is useful in determining the severity of the frost event (Kiesselbach and Ratcliff, 1920); however, the actual rate of freezing and duration of the freezing temperatures are also important. The freezing rate may determine the type of damage. Very fast freezing rates lead to intracellular ice formation, whereas slower freezing rates often cause dehydration associated with extracellular ice formation (Mazur, 1969). Burke et al. (1976) state that the duration of freezing temperature also influenced the degree of damage. Several studies have shown that increasing the duration of the frost or freezing treatment increases damage (Hartwigsen, 1999; Woltz, 2003).

While the freezing rate and process influence frost damage, the thawing rate and period do not appear to enhance or amplify the damage (Rossman, 1949a; Fick, 1989). Hartwigsen (1999) describes the thawing process as a reversal of the freezing process based on a review of literature. The vapor pressure outside of cells is greater than inside the dehydrated cells leading to migration of water back into the cell. The rate of the migration is dependent on the permeability of the plasma membrane and the rate of warming. Repeated freezing and thawing increases the effect of frost damage as compared to only one frost cycle (Burke et al., 1976). Rossman (1949a, 1949b) also showed that repeated frost events increased damage, but found that the first frost event is the most damaging and important.

Thawing may not cause more damage but perhaps a slow thaw may make recovery and repair easier.

#### Freezing and Frost Damage

Freezing can lead to various physical, mechanical, and biochemical changes in seeds. Obvious physical changes in frosted seed lots are malformed and small seeds, mechanically damaged seeds, and seed lots with low test weight (Hurburgh and Benson, 1995). Mechanical and physiological alterations are often associated with the dehydration aspect of freezing. The formation of ice crystals in the intracellular region physically ruptures the cell. Ice crystals in the extracellular region can also damage surrounding cells by either puncturing the cell wall or causing abrasions (Hartwigsen, 1999; Burke et al., 1976). Plasmolysis, or the shrinkage of the protoplast due to water loss from the cell, and cellular collapse can cause extensive mechanical damage. The higher the degree of vacuolation in the cell, the more likely damage due to plasmolysis will occur (Vertucci and Farrant, 1995; Pammenter and Berjak, 2000; Perdomo and Burris, 1998; Farrant et al., 1997). The damage associated with cell collapse and plasmolysis also includes membrane fusion and alteration. The contents of the cell become so packed together that interactions between membranes occur. Normally these interactions would be prevented by water on the membrane surfaces. Endocytotic vesiculation of the plasmalemma decreases the membrane's surface area (Vertucci and Farrant, 1995; Uemura and Steponkus, 1998). Endocytotic vesiculation refers to the vesicles that form from invaginations of the membrane, which ultimately break away from the membrane. Due to the reduction in surface area, the membrane cannot expand upon rehydration causing it to rupture. This process and associated injury is called expansion-induced lysis (Uemura and Steponkus, 1998).

Additional damage to membranes and macromolecules can occur from ice formation in the extracellular region. When the cell becomes dehydrated, water usually bound to the hydrophilic regions of the plasma membrane is unavailable for membrane and macromolecule stabilization. When water is removed to levels below 0.25 g H<sub>2</sub>O/g dm, the liquid crystalline structure of the membrane is no longer

stabilized by the water (Bewley and Black 1994). The lipid bi-layer phase shifts from a liquid crystalline state to a gel state (Hartwigsen, 1999). While the membrane is changing phases, it loses the ability to be selectively permeable and therefore leaks (Hoekstra et al., 1997; Bewley and Black, 1994). As the membrane shifts back to the liquid crystalline state during rehydration, the membrane also loses the ability to be selectively permeable (Hartwigsen, 1999; Hoekstra et al., 1997; Bewley and Black, 1994). Sugars can replace water on the membrane surface during dehydration and prevent the transition to a gel state and stabilize the liquid-crystalline state (Bewley and Black, 1994). This will be discussed in more detail under Freezing Tolerance, Protection, and Response. Lamellar-to-hexagonal<sub>II</sub> phase transitions occur when endomembranes, such as the chloroplast envelope, come in close proximity to the plasma membrane and a protective layer of water is absent (Gordon-Kamm and Steponkus, 1984). This type of phase transition damages both membranes.

Many macromolecules also rely on water stabilization and are irreversibly damaged when water is removed (Koster, 1991). These changes have severe repercussions once the seed is imbibed and the germination process begins. Unregulated metabolism can create harmful free radicals (Vertucci and Farrant, 1995). These free radicals increase seed deterioration (Perdomo and Burris, 1998). The longer unregulated metabolism is allowed to continue, the greater the level of seed deterioration (Vertucci and Farrant, 1995). Dehydration also affects the chemical and electrical properties of the cytoplasm. Electrolytes and other solutes can precipitate out of the cell solution (Mazur, 1969). This leads to detrimental pH and abnormal electrical-charge gradients in cells and across membranes (Steponkus, 1984).

#### Desiccation Tolerance

Desiccation tolerance is the ability for tissue to survive at low moisture contents and subsequently germinate when adequate moisture is available. Seed species that obtain desiccation tolerance during development are classified as orthodox. As orthodox seeds develop, several physiological changes may signal or

induce the acquisition of desiccation tolerance. During this seed maturation phase, insoluble reserve compounds accumulate, the volume of vacuole decreases, additional protective molecules are synthesized, organelles dedifferentiate, seed metabolism slows or ceases, and finally, protective molecules are synthesized (Pammenter and Berjak, 2000). Although this is the general pattern for orthodox seeds, the details of each of these steps varies and may occur in different orders.

Freezing does not adversely affect all seed. Brown and Escombe (1898) report that dry seeds of several species, including oats and barley, can survive very low temperatures for extended periods of time. These seeds can withstand temperatures as low as  $-186^{\circ}\text{C}$  to  $-192^{\circ}\text{C}$  for 110 consecutive hours without exhibiting significant damage or decline in germination.

Kiesselbach and Ratcliff (1920) conducted a multi-year frost study in corn seed. They followed the relationship between seed moisture content and frost damage. The incidence of frost injury in corn seeds varies from year to year, and is dependent on the date when a specific line reaches maturity (Kiesselbach and Ratcliff, 1920). They concluded that the moisture content of the seed at the time of the frost event is less important than the level of seed maturity of a line. Therefore, moisture content of the seeds alone is not sufficient information to determine the possible effect of frost on seed quality. Seed maturity and desiccation tolerance are key factors to consider at the time of a frost, however, they are very difficult to measure. Hartwigsen (1999) and Cordova-Tellez and Burris (2002a) reinforced the concept that high moisture alone is not the cause of damage. The level of maturity and, more importantly, the level of acquisition of desiccation tolerance are critical to the survival of the seed. This supports Rossman's claim (1949a) that physiological changes in the maturing seed, as opposed to the mere decline in moisture content over time, are associated with frost tolerance.

These differences may be the result of the different locations where water is found in seeds and the changes in the role of water as seeds mature. Five hydration levels have been distinguished and characterized in seeds (Vertucci and Farrant, 1995; Pammenter and Berjak, 2000). Hydration level V is above  $700\text{ g H}_2\text{O kg}^{-1}$  dry



matter (dm) and is associated with water potentials above -1.5 MPa. Water at this state is the solvent for dilute solutions within cells. Cell division and expansion can occur and germination can proceed if all other conditions are appropriate. In most species, all other cell functions are normal at these water levels. In maize, this stage occurs very early in seed development before reserve deposition. Hydration level IV is between 450 and 700 g H<sub>2</sub>O kg<sup>-1</sup> dm and is associated with water potentials between -3 and -1.5 MPa. Water is still abundant but solutions are more concentrated than those found at hydration level V. Respiration and other cell functions such as protein and nucleic acid synthesis are active. Both the endosperm and embryo of maize seeds are at this hydration state for most of development, reserve deposition and maturation, until right before physiological maturity. Hydration level III is between 250 and 450 g H<sub>2</sub>O kg<sup>-1</sup> dm and is associated with water potentials between -11 and -3 MPa. Water is found in a glassy state and at the lower water potentials is not freezable (Williams and Leopold, 1989). These glasses may provide some protection from freezing especially at the lower moisture contents (Vertucci, 1989). Respiratory activity is low, but free radicals can still be produced. Maize seeds reach this stage just before physiological maturity and remain at this level until harvest. Hydration level II is between 80 and 250 g H<sub>2</sub>O kg<sup>-1</sup> dm and is associated with water potentials between -150 and -11 MPa. Glasses are still present and only low catabolic activity can be detected. Water is bound to macromolecules and membranes to stabilize their surfaces and, therefore, it does not freeze easily. Hydration level I is below 80 g H<sub>2</sub>O kg<sup>-1</sup> dm and is associated with water potentials less than -150 MPa. Water is only present in tightly bound forms and regular metabolic activity in the seed has slowed below measurable levels or ceased. This tightly bound water does not freeze. Maize seeds are only dehydrated to this state for long-term storage. Below 50 g H<sub>2</sub>O kg<sup>-1</sup> dm seeds deteriorate more rapidly (Bewley and Black, 1994). This may be caused by macromolecule and membrane destabilization when too much bound water is removed.

Liu et al. (1995) showed that, at the same freezing temperature, the amount of unfrozen water in seeds is the same for several moisture contents. However, the

total amount of frozen water varies. The unfrozen water represents the bound water within the cells. Therefore, the amount of bound water in seeds does not vary among several moisture contents. However, the amount of unfrozen water varies depending on freezing temperature. These results indicate that the lower the freezing temperature, the lesser the amount of bound water present in the cells. As temperature decreases, more bound water freezes, increasing the severity of the dehydration stresses. From this study, the conclusion can be made that the amount of water in seeds that is not bound or used can be lost, and the amount of water that can be lost but isn't, can freeze.

#### Freezing Tolerance, Protection, and Response

Soluble carbohydrates are produced as a response to freezing injury. These carbohydrates are also produced during the natural progression of the acquisition of desiccation tolerance in orthodox seeds. Raffinose and stachyose can prevent sucrose crystallization, which in turn allows sucrose in solutions to form a glassy state, thus preventing membrane fusion (Chen and Burris, 1990; Górecki et al., 1997; Hoekstra et al., 1997; Koster, 1991). Sucrose and oligosaccharides can also act as antioxidants to scavenge free radicals (Górecki et al., 1997). The transition from accumulating reducing sugars to accumulating oligosaccharides in orthodox seed may be important in desiccation tolerance because it limits the available sources of free radicals (Vertucci and Farrant, 1995; Górecki et al., 1997). The hydroxyl groups of sugars can help stabilize lipid membranes and macromolecules by replacing surface water (Vertucci and Farrant, 1995; Chen and Burris, 1990; Pammenter et al., 1991). The ratio between sugar and phospholipid mass is important for stabilization to occur (Hoekstra et al., 1997). The ratio of sucrose to raffinose is important in glass formation (Vertucci and Farrant, 1995; Hoekstra et al., 1997). Glass formation prevents cellular collapse, harmful free radicals from rapidly spreading, prevents further rapid loss of water from the cell, and continues to stabilize membranes and macromolecules via hydrogen bonds (Koster, 1991). Therefore, all cells are kept in a stasis-like condition until rehydration occurs (Vertucci and Farrant, 1995; Pammenter and Berjak, 2000). Many orthodox seed

species form glasses upon dehydration. However, some recalcitrant species also have glassy states. In these cases, a glassy state does not indicate desiccation tolerance for recalcitrant species (Pammenter and Berjak, 2000). Sucrose and raffinose are the most prominent soluble carbohydrates when plant tissue is exposed to low temperatures or is dehydrated (Chen and Burris, 1990; Hartwigsen, 1999). These carbohydrates may be used for the synthesis of other protective proteins and enzymes (Hughes and Dunn, 1996).

There are several other protective mechanisms in seeds that minimize the impact of dehydration and freezing at a cellular level. One of these mechanisms is the migration of lipid bodies. Lipid bodies migrate to the perimeter of cells during maturation drying (Cordova-Tellez and Burris, 2002; Perdomo and Burris, 1998). In this study, decreased drying damage was associated with alignment of lipid bodies along the plasma membrane. Lipid bodies protect the plasma membrane by slowing dehydration and allowing expansion during rehydration. As lipid bodies align along the plasma membrane, the surface area of the cell which can lose water decreases. The authors hypothesized that water was lost more slowly and in a more organized fashion (Cordova-Tellez and Burris, 2002). Also, the lipid bodies represent a reserve of membrane materials that can be used upon rehydration when the membrane expands. This expansion is critical for preventing expansion-induced lysis (Vertucci and Farrant, 1995; Uemura and Steponkus, 1998).

Amphipathic compounds also migrate to the plasma membrane during dehydration (Hoekstra et al., 1997). The amphipathic compounds keep the membrane fluidized and prevent phase transitions from occurring (Pammenter and Berjak, 2000). The presence of amphipathic compounds can allow additional leakage of solutes upon rehydration (Golovina et al., 1998). However, the benefits of the protection they provide to the membrane outweigh the negative impact of this leakage. Flavonols are one such class of compounds. These amphipathic compounds enter the membrane when the cell undergoes desiccation (Hoekstra et al., 1997), and act as antioxidants protecting the membrane from oxidative degradation (Golovina et al., 1998).

Antioxidants are an important class of cell and tissue protectants. If a low water content seed is to remain viable for long periods of time, it is important that there is a mechanism for coping with the products of catabolic reactions.

Antioxidants scavenge for free radicals produced during metabolism (Vertucci and Farrant, 1995; Golovina et al., 1998). There are other compounds within the cytosol that act as antioxidants. Tocopherol, sucrose and phytate are three examples (Vertucci and Farrant, 1995).

There are many protein groups associated with plant responses to freezing and thawing. Lipid transfer proteins (LTP) are important in the transfer of lipids across membranes and may also be important in wax synthesis and secretion (Hughes and Dunn, 1996). The synthesis of dehydrins, a group of proteins usually associated with dehydration, are also induced by freezing (Close et al., 1989). Although dehydrins are expressed in many plant species under a wide range of environmental conditions, the general composition of the proteins are very similar (Hartwigsen, 1999). Dehydrins are one category of late embryogenesis abundant (LEA) proteins. LEA's are thought to stabilize macromolecules and membranes during desiccation (Close, 1997). Another group of proteins upregulated in response to freezing are the cold-regulated (COR) proteins (Steponkus 1998). These COR proteins are also similar to dehydrins.

#### Genetic Component

The genetic background of a line or hybrid plays a role in freezing tolerance. The maternal parent is much more influential than the paternal parent (Rossman, 1949a). He speculated that the stronger maternal influence is related to the endosperm characteristics rather than the pericarp. However, Woltz (2003) found that endosperm characteristics (e.g. sugary or starchy endosperm), do not influence viability or vigor after freezing. Husk protection is also a maternal trait, but its importance in preventing freezing injury is not fully understood. It is known that the husk acts as an insulator and "buffers" temperature changes (Rossman, 1949a; Fick 1989). However, there is not an apparent relationship between husk insulation and decreased injury (Rossman, 1949a). The husk is the most important maternal tissue

impacting ice formation in the ear. Fick (1989) hypothesized that the husk may be an ice nucleation source. In his study, ears surrounded by husks but with the vascular connections between the shank and the husks severed responded similarly to ears with husks completely intact. Therefore, ice formation did not proceed from the husk to the ear via the vascular tissue. If the husk is the ice nucleation point, as previously proposed, ice propagation would move through the husk layers to the ear. He found that the extent of damage in ears frozen with the husks intact is the same as ears still attached to the mother plant. Removing the husks before freezing the ear decreased seed vigor (Fick, 1989). Woltz (2003) confirmed that detaching the ear from the plant does not increase frost damage as long as the husks are intact.

#### Effects of Drying Frost-Damaged Seed

Rossmann (1949b) reports that drying freeze damaged seeds slowly to an acceptable storage moisture contents is less damaging than rapid drying. When seeds are dried too fast, protective mechanisms in the cells do not have time to initiate (Pammenter et al., 1998). However, when seeds are dried too slowly, the unregulated metabolism of the damaged seeds is allowed to continue and seed deterioration increases (Vertucci and Farrant, 1995; Pammenter et al., 1998; Pammenter and Berjak, 2000). Therefore, a middle ground must be found where unregulated metabolism is prevented but the initiation of protective mechanisms is allowed.

Cordova-Tellez and Burris (2002b) show that immature maize seed can be safely harvested and desiccated if the drying rate is slow or it is preceded by a preconditioning treatment. Preconditioning implies maintaining the seeds at a slow drying rate for a period of time prior to rapid drying. The preconditioning or slow drying may allow the acquisition of desiccation tolerance through the alignment of lipid bodies along the plasma membrane (Cordova-Tellez and Burris, 2002a). This lipid body migration to the internal surface of the cell membrane is associated with better seed quality. Exposing seeds to a fast drying rate following the preconditioning treatment preserves cell structures and prevents detrimental reactions to continue (Pammenter et al., 1991). The conclusion drawn by these

researchers is that a preconditioning drying can simulate the natural maturation process on the parent plant, thus allowing the onset of desiccation tolerance (Hong and Ellis, 1997; Perdomo and Burris, 1998). Immature seeds exposed to a fast drying rate without prior preconditioning suffer a loss in seed quality and viability (Hong and Ellis, 1997; Cordova-Tellez and Burris, 2002b).

#### Monitoring a Freezing Event

Thermocouples are one of the most common tools for monitoring temperature in plant tissue. Several studies have used thermocouples both in vegetative tissues (Wisniewski et al., 1997; Räisänen et al., 2006) and seeds (Fick, 1989; Woltz et al., 2005) to determine the supercooling and freezing points. The supercooling point is the minimum temperature reached by a liquid prior to ice formation. Once ice nucleation occurs, an exothermic peak results from the release of the latent heat of fusion. The highest temperature reached at the top of this peak is the freezing point. The characteristics of the exotherm peak, as well as the time periods directly surrounding the peaks, provide insights into the physical and physiological effects of freezing seed tissue.

Thermocouples do not determine ice nucleation points; they simply measure the temperature of the plant tissue. There are two reasons why thermocouples cannot accurately determine the ice nucleation point in plant tissue. First, ice nucleation is relatively random and it would be nearly impossible to randomly find the nucleation site (Fick, 1989). The second reason is that the thermocouple itself can induce ice nucleation. The metal wire of the thermocouple itself or its shield cools at a faster rate than plant tissue and will “frost” first, acting as an ice nucleation point for ice formation within the plant tissue (Burke et al., 1976; Fick, 1989). For these reasons thermocouples are limited in the information they can provide; however, they are the best technology available for determining temperatures in seeds.

Previous studies have used thermocouples to measure internal kernel temperature and provide a basic understanding of maize freezing characteristics. Fick (1989) determined that the supercooling point of maize seeds on the ear is

between -4 and -2 °C. Fick (1989) also confirmed previous observations that the temperature at which ice nucleation occurs determines the degree of damage to the tissue (Rossman, 1949b; Burke et al., 1976). Supercooling without subsequent ice nucleation may or may not cause damage. Increased damage to tissue is associated with lower supercooling points prior to ice nucleation (Burke et al, 1976). Thus, tissue supercooled to -3°C without ice nucleation may or may not suffer damage. However, the extent of the damage will be more severe in seed tissue supercooled to -6°C and followed by ice nucleation, than when supercooled to -3°C followed by ice nucleation.

The rate at which the tissue is supercooled is also important. Supercooling slowly or allowing more time before ice nucleation may be advantageous to the seed. During a natural frost event, slower supercooling rates may allow the plant tissue to avoid freezing. Woltz (2003) observed a genotypic effect on freezing characteristics of corn seeds,. However, general freezing patterns exist among all genotypes. As seeds approach physiological maturity and seed moisture content decreases, the freezing point also decreases. At very low moisture contents, exothermic peaks may not be detectable because there is not enough water available to freeze and release measurable heat (Woltz, 2003). This study also explores the freezing point differences between the embryo and endosperm tissues. As seeds matured, the lower supercooling point of the endosperm is associated with the lower moisture content of the endosperm compared to the moisture content of the embryo. These studies provide many details on freezing characteristics of maize seeds, and pave the way for future research.

### Seed Testing

Seed damage, including damage caused by freezing, leads to decreased seed vigor, decreased viability, and increased electrolyte leakage. Kiessellbach and Ratcliff (1920) found that planting frosted seed does not reduce yields compared to non-frosted seed if the same field stand can be established. If the seed is able to survive, germinate, and develop into a healthy normal seedling there is no residual impact from frost. The damages associated with frost lead to changes in seed

viability and vigor. Therefore, common seed quality tests can be used to identify frost damage.

Several seed quality tests expose seeds to various stresses before and/or during germination. The cold test along with all its alternative protocols is probably the most common vigor test used for seed corn in the Midwest. All cold tests have three common stress factors: low temperature, usually around 10 °C; excessive moisture, enough for exposing the embryo to nearly anaerobic conditions; and non-sterile soil as a source of soil-pathogen inoculation (Burris and Navratil, 1979). Cold tests are used to predict early field emergence, however, the methods and degree of correlation to field emergence vary greatly (Burris and Navratil, 1979). The saturated cold test is considered the most stressful cold test, but is the most commonly used by seed companies (Gutormsen, 1996). In the saturated cold test, the embryo is placed down into the soil and water which leads to respiratory stress due to limited gas exchange (Hoegemeyer and Gutormsen, 2000). At the same time CO<sub>2</sub>, ethanol, and acetaldehyde accumulate in the seed and surrounding media (Martin et al., 1988; Hoegemeyer and Gutormsen, 2000). Martin et al. (1991) suggested that CO<sub>2</sub> inhibits germination in corn seeds subjected to soaking prior to germination. They proposed that, although the saturated cold test is more time consuming than the soak test, it has a higher correlation to field emergence than the soak test (Martin et al., 1988). Martin et al. (1988) speculated that the differences between the saturated cold test and the soak test may have originated from damage caused when seed was over dried prior to storage. The authors concluded that lower moisture seed is more sensitive to chilling injury, which decreased saturated cold test germination compared to the soak test.

Accelerated aging test is a relatively simple seed vigor test; however, its use in seed corn has been highly debated. Originally the accelerated aging test was developed to predict storability of a seed lot (Delouche and Baskin, 1973). However, the results have been correlated to field emergence (TeKrony and Egli, 1977; Egli and TeKrony, 1996). There are three stress variables to the accelerated aging test: increased temperature; nearly 100% relative humidity; and time. These conditions



lead to increased respiration and metabolism, during which low vigor seeds deteriorate rapidly (McDonald, 1998).

Frost damaged seed lots have increased leakage of solutes during seed imbibition (Hartwigsen, 1999). This change in seed leakage typically is measured using the electrical conductivity test. However, this test is not commonly used by the seed industry. Electrical conductivity values are lower in the treatments where the husks are left intact (Fick, 1989).

The suitability of these common seed quality tests (SC, Soak, and AA) for measuring frost damage in maize seeds has been explored. Fick (1989) reported that the results of the standard germination test of frozen seed lots are not significantly different from those of the sterile cold test. Woltz (2003) found that frost damaged seed lots respond differently when tested in the cold test and the accelerated aging test. In these experiments, the standard germination and accelerated aging tests are significantly different for seeds frozen at early maturities, while the cold test results are not. Moreover, the electrical conductivity test results provide little additional information for identifying or measuring frost damage. This indicates that seed quality tests may vary in their ability to measure or detect frost damage.

The tetrazolium test is traditionally used for determining seed viability, although its use for seed vigor determination is outlined in the Association of Official Seed Analysts (AOSA) Tetrazolium Testing Handbook (AOSA, 2002) and Seed Vigor Testing Handbook (AOSA, 2002). The tetrazolium test is often considered the most accurate test for early determination of frost damage in seed corn. This test requires trained analysts and more labor than traditional germination tests, but it leads to faster results. Seed damage can be assessed in 24 hours using the tetrazolium test rather than waiting 7 to 8 days for the results of a germination test. Although the tetrazolium test is the first tool for identifying frost damage, germination tests should be used for evaluating subsequent damage in storage (Goodsell, 1948). The results obtained with the tetrazolium test must correlate to either the standard germination test or a vigor test such as the cold test to be a valuable tool in seed

testing. The traditional staining procedure outlined in the Tetrazolium Testing Handbook (AOSA, 2000) states that seeds should be imbibed overnight, bisected longitudinally through the embryo, placed in 0.1% tetrazolium chloride solution, and rinsed with water before evaluation. However, the traditional staining method is inadequate for accurately assessing frost damage. The embryos are stained dark, and staining patterns are difficult to differentiate. The vigor categories of seed stained by the traditional method, especially high and medium vigor, are too similar to separate.

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## **CHAPTER 2. DETERMINING THE EXTENT OF FROST DAMAGE IN MAIZE SEED USING THE TETRAZOLIUM TEST**

A paper published in Crop Management<sup>1</sup>

### **Abstract**

Frost damage in seed corn causes a significant decrease in seed quality. The tetrazolium (TZ) test has been used to identify types of physiological damage in several plant species, including frost damage in corn. Quantifying the amount of seed damaged by frost and the consequent loss of seed vigor are important for making management decisions. The current tetrazolium test procedures provide seed viability information but do not estimate seed vigor. In our project, the tetrazolium staining procedure was modified to allow separation of viable seeds into vigor categories. Vigor categories were defined based on Association of Official Seed Analysts (AOSA) recommendations, previous research, and current observations. The results from the tetrazolium test were compared with standard germination and saturated cold tests during the first 6 months of storage. The staining procedure aided in visual identification of frost damage. Although a strong correlation was found between seed viability as determined by the tetrazolium test, the standard germination test, and the saturated cold test, sorting seeds into vigor categories in the tetrazolium test did not improve the correlation between the tetrazolium test and the saturated cold test.

### **Introduction**

Frost damage is costly to seed producers for two reasons. First, the seed decreases in value because of physiological damage and second, the extent of damage can only be assessed after seed harvest, drying, conditioning, and storage of damaged seed. The delay in assessing seed deterioration is caused by the

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initially slow decline in seed vigor and viability. The tetrazolium test is traditionally used for determining viability, although its use for seed vigor determination is outlined in the Tetrazolium Testing Handbook [1] and Seed Vigor Testing Handbook [3]. The tetrazolium test is often considered the most accurate test for early determination of frost damage in seed corn. This test requires trained analysts and more labor than traditional germination tests, but it leads to faster results. Seed damage can be assessed in 24 hours using the tetrazolium test rather than waiting 7 to 8 days for the results of a germination test. Although the tetrazolium test is the first tool for identifying frost damage, germination tests should be used for evaluating subsequent damage in storage [5]. The results obtained with the tetrazolium test must correlate to either the standard germination test or a vigor test such as the cold test to be a valuable tool in seed testing.

The use of mechanical detection systems for evaluating tetrazolium -stained seeds has been explored [6, 12]; however, only the color ratio was measured. Scanning tools were used to calculate the ratio of red-stained tissue to white-unstained tissue. This value was termed the tetrazolium staining ratio and was used to determine the viability of the seed. However, the location of staining patterns is as critical as the staining intensity [4]. The precision of these mechanical evaluations is reduced because the location of stained and unstained tissue is not taken into account. Xie and Paulsen [12] increased the accuracy of evaluation by creating a larger algorithm. Their algorithm also accounts for the size of the unstained area based on the assumption that the larger the unstained area, the greater the likelihood that the seed is nonviable. However, they were unable to separate critical locations of unstained areas such as the radicle, plumule, and seminal roots from the entire embryo. This inability to identify staining patterns in critical regions limits the application of mechanical detection for diagnostic evaluations. Therefore, skilled analysts are still necessary for interpreting the tetrazolium test for vigor determination.

The traditional staining procedure outlined in the Association of Official Seed Analysts (AOSA) Tetrazolium Testing Handbook [1] states that seeds should be

imbibed overnight, bisected longitudinally through the embryo, placed in 0.1% tetrazolium chloride solution, and rinsed with water before evaluation. However, the traditional staining method is inadequate for accurately assessing frost damage. The embryos are stained dark, and staining patterns are difficult to differentiate. The vigor categories of seed stained by the traditional method, especially high and medium vigor, are too similar to separate. We feel that it is important to develop an improved staining method for the tetrazolium test and to clearly define vigor categories for this test specifically relating to frost damage. By improving the identification of frost damage in seed corn, seed producers and brokers have a better tool for making management decisions.

The objective of our research was to develop a consistent tetrazolium staining method and evaluation criteria for separating frosted seed into vigor categories. Additionally, we wanted to compare the numbers of seeds in these vigor categories to results from the standard germination and saturated cold tests.

### **Production of Frost-Damaged Seed Corn**

Hybrids B73xIRF311 and Mo17xIRF311 were produced in isolation in 2003 and 2004 near Ames, IA. These two female lines were chosen to represent different genotypic tolerance to cold temperatures. B73 was developed by the federal-university corn breeding program at Iowa State University and is considered relatively tolerant to cold temperatures. Mo17 was developed by the federal corn breeding program at University of Missouri and is considered relatively sensitive to cold temperatures. IRF311 pollinator is from the Iowa Research Foundation and was selected based on nick with B73 and Mo17. Samples were harvested at three moisture content ranges; 30-35%, 40-45%, and 50-55% moisture content fresh weight (fw) basis. Two field replications were used for this experiment. Ears were harvested with the husks and shank intact. Immediately following harvest, all ears were placed in a cold room at 50°F for a minimum of 4 hours to ensure uniform cooling rates. Control samples were then husked and dried, while remaining ears were frozen in a Conviron growth chamber (Controlled Environment Limited,

Winnipeg, Manitoba, Canada). The frost cycle lasted 24 hours, beginning and ending at 50°F and including a minimum of 8 hours below 32°F and 2 hours at the minimum temperature 23°F. After the frost treatment, ears were husked and dried. Samples were dried on the ear in experimental sized forced air driers at a temperature below 90°F. All samples were shelled using an experimental sheller and stored in a cold room (50°F at 50% RH) until the time of testing.

Additional seed samples were harvested after the first natural frost event recorded on 2 October 2004. Data from these naturally frosted samples were included in the study as a comparison. However, seeds had reached physiological maturity (as determined by black layer formation) in the field before a natural frost; therefore, all naturally frosted samples were below 35% moisture fw at harvest. These samples were dried, shelled, and stored in the same manner as the artificially frosted seed samples.

Tetrazolium tests were conducted starting after all samples were shelled and were repeated every 6.5 weeks for 6 months. One hundred seeds were placed in moistened paper towels for 12 hours to hydrate. Seeds were bisected longitudinally through the embryonic axis. One-half of each seed was selected for evaluation. The selected half was placed with the cut surface on the moist paper towel until the entire sample had been cut. The seeds were then soaked in water for 10 minutes. After this time had elapsed, the water was drained and replaced with 0.1% tetrazolium chloride solution (Sigma-Aldrich, St. Louis, MO). The seeds were allowed to stain for 1 hour and 10 minutes at room temperature. After staining, the tetrazolium solution was drained and seeds were rinsed. Samples were either evaluated immediately or returned to water and placed in a refrigerator for a maximum of 2 hours before evaluation.

Evaluation was done under a stereomicroscope (Fischer Scientific, Pittsburgh, PA). Seeds were placed into one of four categories based on the guidelines for the tetrazolium test as a vigor test, AOSA Seed Vigor Testing Handbook [3], and the criteria used by Moore and Smith [11]. Instead of using five viable categories as described by Moore and Goodsell [10], only three were used in

keeping with the AOSA recommendations. Some additional observations were recorded during evaluation to assist with the identification of frost damage.

The standard germination (WG) test was conducted as described in the AOSA Rules for Testing Seeds [2] with the exception that only 100 seeds per seed lot were tested. Saturated cold (SC) test was conducted as described by Martin et al. [7] with the following changes. After 7 days at 10°C, the trays were moved to 25°C and alternating dark/light cycles of 4 hours each for 4 days. Only 100 seeds per seed lot were used. Evaluation of seedlings was completed according the AOSA Rules for Testing Seeds [2]. The standard germination test and the saturated cold test were done every 6.5 weeks for the first 6 months of storage.

### **Description of Vigor Categories**

Previous work outlines methods for determining seed vigor using the tetrazolium test. Previous researchers have suggested alternative methods for evaluating seed damage, but the protocol and evaluation criteria were not clearly defined. Moore and Goodsell [10] found that their top three vigor categories (out of five) in the tetrazolium test were correlated to the cold test. The difference between Moore and Goodsell's third and fourth vigor categories was that the embryos in the third category only showed beginning signs of deterioration at the cut surface. In the third category, the underlying tissues were stained normally and in the fourth category the degradation was more pronounced and the underlying tissues were deeply bruised and water soaked. We used similar criteria to designate between our medium and low vigor seed categories. If water soaking was present below the cut surface, the seed was categorized as low vigor. However, seeds that appeared watery or bruised in the plumule region were considered low vigor seeds independent of the underlying tissue.

The four categories are high vigor, medium vigor, low vigor, and nonviable. For seeds to be placed into the high vigor category (Figure 2), all critical embryo tissues must be intact and functional. Critical embryo tissues are shown in Figure 1. The predominant staining pattern is red spots on a white or slightly pink background.

Slight damage may be found in the scutellum, but the remaining tissue is healthy. Staining patterns in damaged seeds can be classified into two distinct groups. In the first group, stained tissues have a higher red-to-white ratio. In the second group, stained tissues show patterns of concentric rings of white and red or purple indicating ice formation and injury. Moore [9] found that points of injury can be detected before changes in the standard germination test percentage and seed death because the tetrazolium test evaluates individual seed structures, which may vary in soundness and physiological integrity [8].

The seeds placed in the medium vigor category (Figure 3) have visible damage to the embryonic axis, but not severe enough to cause death and consequently lack of staining. The plumule tissue is intense red or has high red-to-white ratio, but rings or bruises are not visible. Bruising, evident by purple tissue and a watery appearance, may be observed in the radicle. However the meristem and majority of the plumule are still healthy. The mesocotyl region may have an intense red color (Figure 3a), but it is not watery or purple. The seeds placed in the low vigor category (Figure 4) have extensive damage to the meristem and plumule tissues. Critical embryo structures are still alive as evident by staining. Staining color is a deep red to purple. Seeds included in this category are germinable, but may produce abnormal seedlings when planted in a vigor test. The embryo may be misshapen as seen in Figure 4b. Seeds in the high, medium, and low vigor categories are all germinable seeds. The final category consists of seeds that are ungerminable or nonviable. Ungerminable seeds are those that lack staining in one or more critical embryo structures. Either the plumule including the meristem (Figure 5a), or the radicle (Figure 5b), or more than one-third of the scutellum (Figure 5c) must be dead and unstained. Some seeds have completely unstained embryos where both the plumule and radicle tissue are dead. The characteristic staining pattern of frost-killed seeds is represented in Figure 6 where the embryonic axis is unstained, but the scutellum stained deep red with white areas.

## **Relationships among Tetrazolium, Standard Germination, and Saturated Cold Tests**

Tetrazolium test categories can be used to predict germination or vigor after several months of storage. Pearson correlation coefficients were calculated between one or more seed vigor categories from the tetrazolium test and the saturated cold test results at 3 and 6 months. The number of viable seeds in the tetrazolium test has a positive correlation ( $r = 0.86$ ) to the percentage of normal seedlings in the saturated cold test after 6 months of storage (Figure 7). Although the nonfrosted control samples are clustered at the top of the graph where germination and vigor are high, the correlation coefficients of the nonfrosted ( $r = 0.61$ ), naturally frosted ( $r = 0.97$ ), and artificially frosted ( $r = 0.93$ ) samples are all significant ( $P < 0.05$ ). Samples exposed to a natural frost were more mature when the frost occurred; therefore, they do not exhibit as much damage as the artificially frosted samples that were frosted at higher moisture contents. There were a few samples with low saturated cold test percentages (<40%). The positive correlation between the saturated cold test results after 3 and 6 months of storage and the tetrazolium test conducted immediately after the frost still exists when the data points with saturated cold test results <40% are removed from the analysis. More importantly, the correlation coefficients of the frost-damaged samples and nonfrosted samples remained similar to each other. The number of seeds in the high vigor category in the tetrazolium test at 0 months does not significantly correlate to the number of normal seedlings in the saturated cold test at any of the testing times (data not shown). Significant positive correlations ( $P < 0.05$ ) were found between the standard germination and saturated cold test percentages compared with the total number of seeds in the high and medium vigor categories of the tetrazolium test (Table 2). However, the correlation coefficients improve when all viable categories (high, medium, and low vigor) are added together. The correlation coefficients and  $P$  values improve for both tests over all time periods (Table 2). This improved correlation indicates that seeds in the low vigor category are still able to produce normal seedlings in the saturated cold test after 6 months of storage in a cold room. Seeds in which only the plumule or radicle

was dead and watery were recorded separately. Adding these seeds to the viable category did not improve the correlations, indicating that these “half-dead” seeds either produce abnormal seedlings or are unable to germinate.

## **Conclusions**

We conclude that the new staining procedure facilitates the evaluation and interpretation of staining patterns in frost-damaged seeds. Classifying viable seeds into separate vigor classes can be beneficial for research purposes or as a diagnostic tool, but it is not necessary for obtaining good estimates of germination or vigor after 6 months of storage.

## **Acknowledgements**

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Table 1. General description of the vigor categories based on the staining patterns of critical embryo tissues.

Category	Plumule Appearance	Radicle Appearance	Scutellum Appearance
High Vigor	Red or pink spots on a white background	Red or pink spots on a white background	Red spots on a pink or white background, may have regions of more intense red
Medium Vigor	Red or intense-red tissue	Red or intense-red tissue, may have purple tint or a watery appearance	Red or intense-red tissue, may have bruising or concentric rings
Low Vigor	Red to purple tissue with a watery appearance	Red or intense-red tissue, may have purple tint or a watery appearance	Red or intense-red tissue, may have bruising, concentric rings, or unstained areas less than 1/3 the scutellum
Ungerminable/ Unviable	Unstained, red, or purple tissue with a watery appearance	Unstained, red, or purple tissue with a watery appearance	Red or intense-red tissue, may have bruising, concentric rings, or unstained areas

Table 2. Pearson's Correlation Coefficients between the TZ test at 0 months of storage and the standard germination test and saturated cold test after 0, 3, and 6 months of storage.

	0 months		3 months		6 months	
	WG	SC	WG	SC	WG	SC
HML of TZ test						
at 0 months	0.68***	0.77***	0.77***	0.89***	0.77***	0.88***
HM of TZ test						
at 0 months	0.48 **	0.44*	0.57**	0.55**	0.49**	0.60**

\*, \*\*, \*\*\* Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

Figure 1. A high vigor seed as indicated by the staining pattern of red spots on a white background. All critical seed structures are stained, and there is no damage present in the embryonic axis.



Figure 2. Critical embryo structures seen in a longitudinally bisected corn kernel: scutellum/cotyledon (a), coleoptile (b), plumule/shoot (c), meristem (d), mesocotyl region (e), seminal root (f), radicle/root (g), coleorhiza (h), and black layer (i).

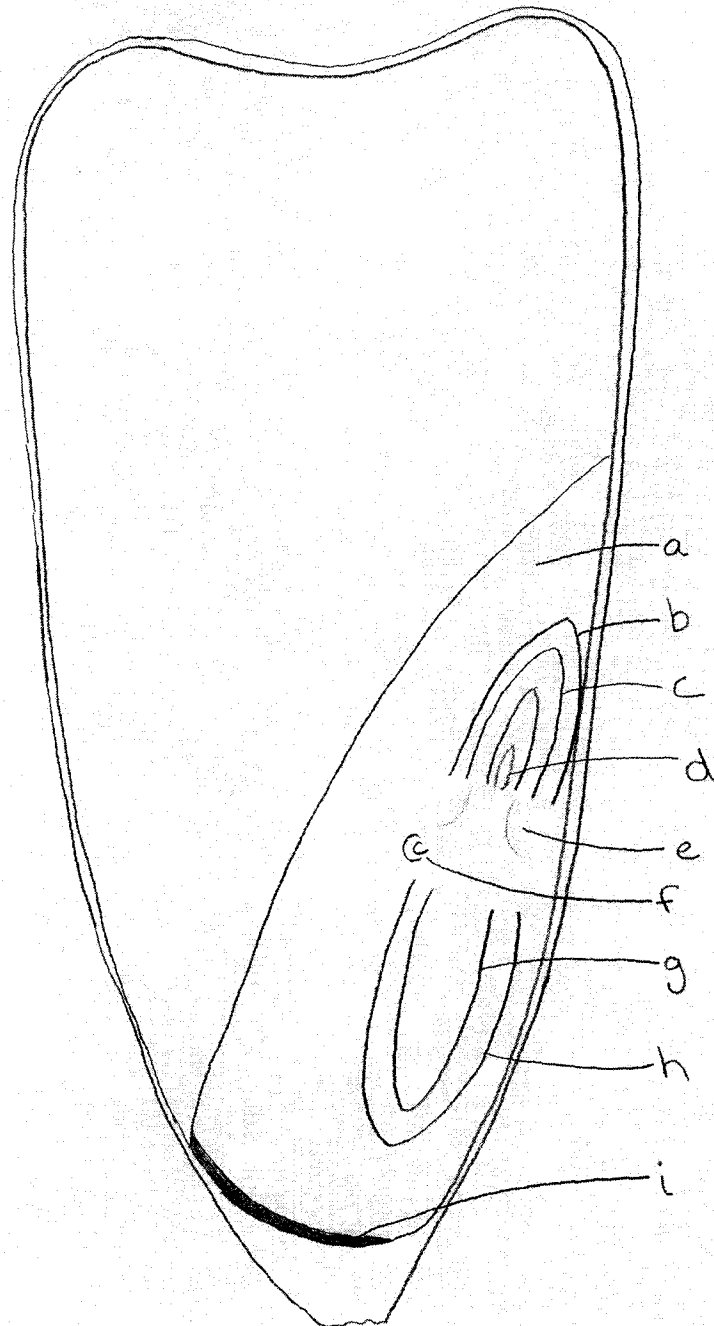


Figure 3. Medium vigor seeds with beginning signs of damage. Red-to-white ratio is much higher especially in the embryonic axis (a). Some seeds in this category will have a healthy scutellum (b), but the intense red staining of the plumule will classify them as medium vigor.

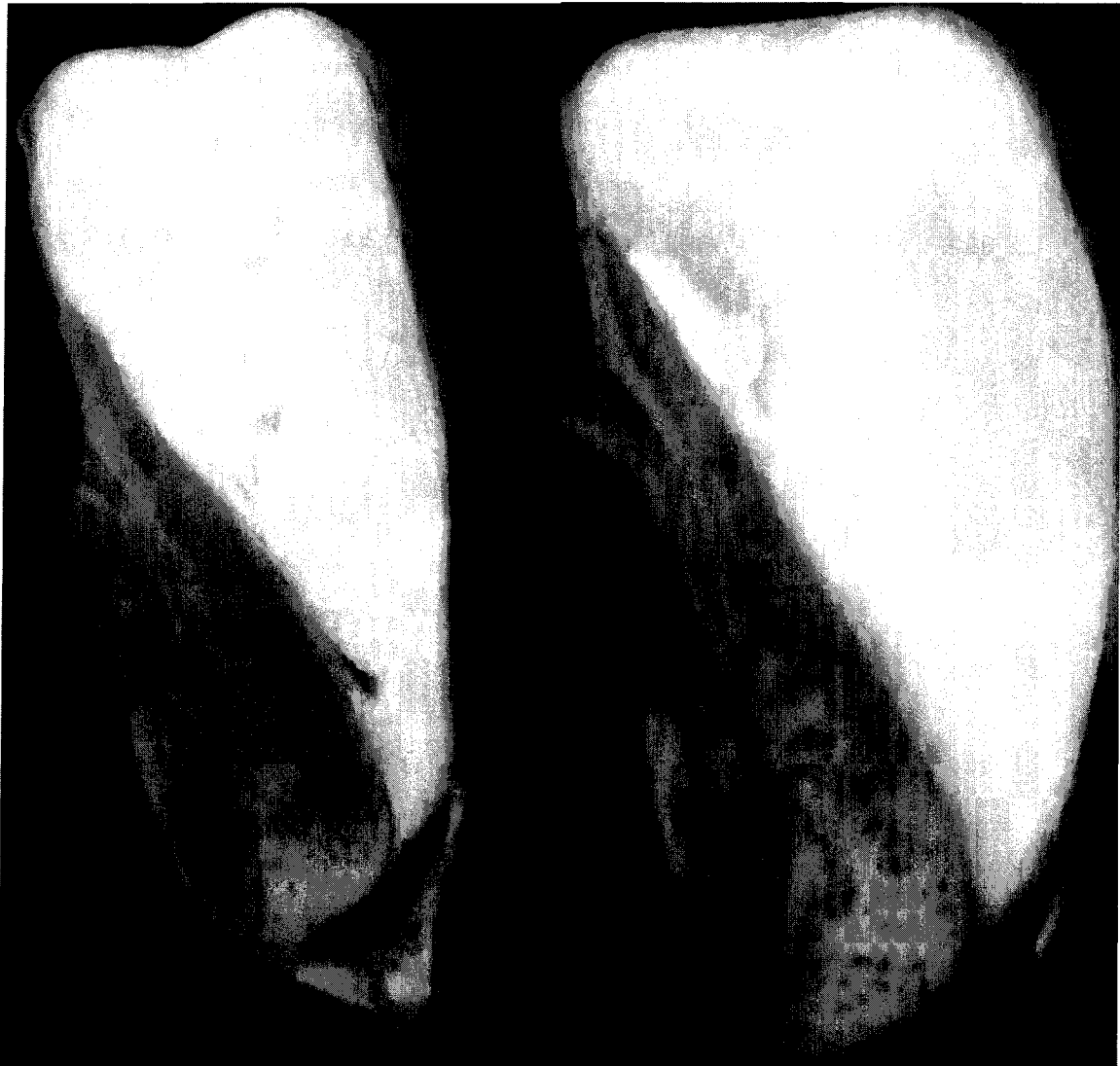


Figure 4. Low vigor seeds with extensive damage to the plumule. Plumule and radicle have a slightly watery or purple-tinted appearance (a). Extreme damage can be seen as deep purple-stained tissue (b). Areas of the scutellum are often bruised as seen in b near the base of the scutellum.

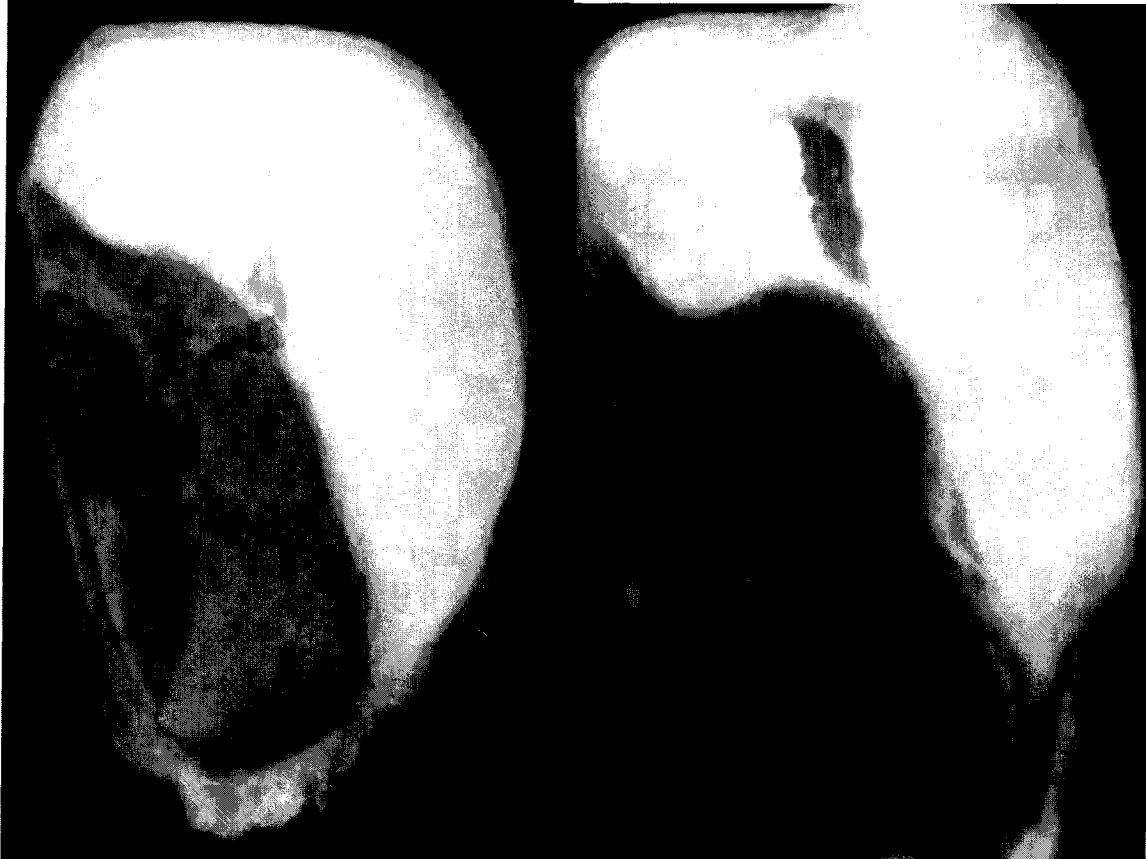


Figure 5. Ungerminable seeds as indicated by the absence of stained tissue in critical embryo structures. Plumule and meristem unstained (a). Radicle unstained (b). Unstained radicle and more than 1/3 of the scutellum unstained (c).

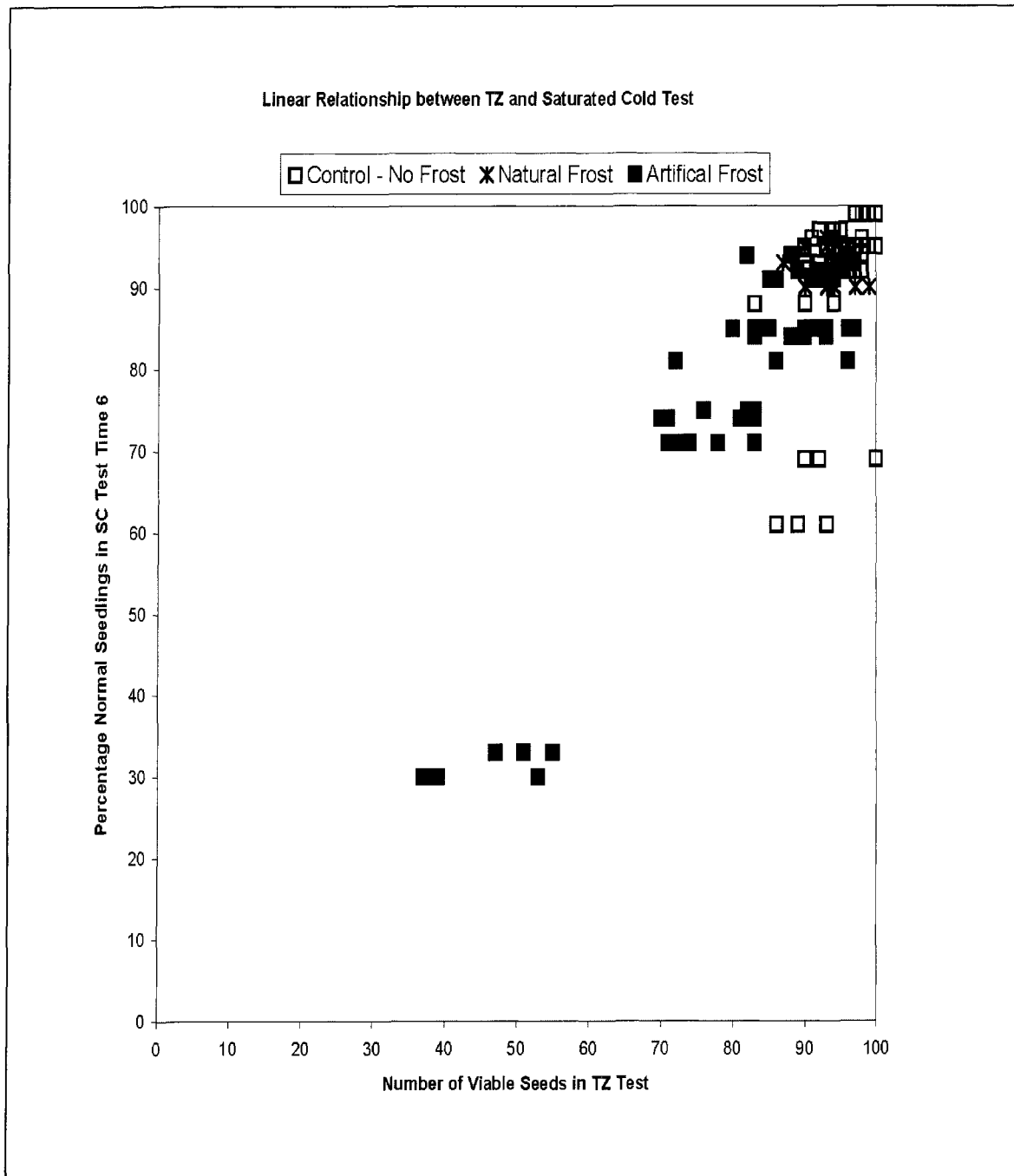


Figure 6. Typical frost-killed seed where the plumule, meristem, and center of the radicle are unstained, whereas all remaining tissues exhibit little damage.





Figure 7. Positive correlation ( $r = 0.86^{***}$ ) between TZ test results and the percentage of normal seedlings in the saturated cold test after 6 months of storage.



## **CHAPTER 3. SEED QUALITY TESTS FOR EARLY DETECTION OF FALL FROST DAMAGE IN IMMATURE MAIZE SEED**

A paper to be submitted to Crop Science

Mindy DeVries and A. Susana Goggi

### **Abstract**

Maize seed production can be severely impacted by a fall frost event because it decreases seed quality. It is important to detect frost damage early and to make marketing decisions before the seed lots are conditioned for sale. This study compared several seed quality tests (standard germination [WG], accelerated aging [AA], saturated cold [SC], and soak tests) for their ability to quantify frost damage. Additionally, these tests were used to predict field emergence in poor and average to good field conditions. Two genotypes (B73xIRF311 and Mo17xIRF311) were harvested at three moisture contents (300-350, 400-450, and 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw). An artificial frost treatment was applied to the seed and frost damage was determined by testing seeds after 0, 1.5, 3, 4.5 and 6 mo of storage. The artificial frost treatment significantly decreased viability and vigor of all the seed lots except for the Mo17 hybrid harvested at 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw. As seeds matured, the damage associated with frost treatment decreased. Laboratory tests did not accurately predict field emergence of frost damaged seed in poor field conditions; however, AA at 0 mo, SC at 0, 1.5, and 3 mo, and soak at 0, 1.5, and 3 mo all had a strong relationship to field emergence in average to good field conditions. Frost damage in a seed lots can be quantified using the SC and soak tests during the first 3 mo of storage and both tests are equal in their ability to predict field emergence.

## Introduction

Fall frost damage is a major threat to maize seed production in the central U.S and is common in the Corn Belt. In the upper Midwest, significant frost damage in seeds occurs once every five years (Burris and Knittle, 1985). Freezing can lead to various physical, mechanical, biochemical, and physiological changes. These changes lead to decreased germination, decreased vigor and increased electrolyte leakage. Early detection of frost damage allows marketing decisions to be made in a timely fashion.

Freezing does not have the same adverse effect on all seed. Some of the earliest freezing studies showed that mature dry seeds of several species, including oats and barley, can survive temperature as low as  $-186^{\circ}\text{C}$  to  $-192^{\circ}\text{C}$  for 110 consecutive hours without significant damage or decline in germination (Brown and Escombe, 1898). Kiesselbach and Ratcliff (1920) conducted extensive experiments on frost events in seed corn fields and the resulting damage. They noted that the degree of maturation observed at specific dates for each line varied from year to year. The degree of plant and seed maturity influenced the response to frost. Kiesselbach and Ratcliff (1920) concluded that moisture content alone is not an adequate predictor of the potential effect of frost. The level of maturation achieved prior to freezing is a key concept, but it is very difficult to measure. Hartwigsen (1999) confirmed that high moisture alone is not the cause of freezing damage. The lack of maturity, and more importantly the lack of acquisition of desiccation tolerance, are the critical factors associated with freezing injury in seed corn. These results support Rossman's claim (1949a) that physiological changes occurring in the maturing seed, rather than the mere decline in moisture content, are important in conferring frost tolerance.

These differences may be the result of the different locations where water is found in seeds and the changes in the role of water as seeds mature. Five hydration levels have been distinguished and characterized in seeds, based on the water content of the tissue and how tightly water is bound to membranes and macromolecules (Vertucci and Farrant, 1995; Pammenter and Berjak, 2000).

Hydration level V contains the most water and cell metabolism is highest. Seeds are at hydration level V very early in development. As seeds mature water content decreases and cell metabolism also decreases. Maize seeds are often stored at lower hydration levels (e.g. levels I and II). The location of water found in “dry” seeds is typically bound to membrane and macromolecule surfaces which stabilizes these compounds.

Liu et al. (1995) showed that the amount of unfrozen water in seeds is the same at the same freezing temperature, regardless of the initial moisture content of the seed. The unfrozen water represents the bound water within the cells. This amount of bound water in seeds does not vary among several moisture contents. However, the amount of frozen water changes with the moisture content. Liu et al. (1995) also observed that the amount of unfrozen water varies depending on freezing temperature. As temperature decreases, more bound water freezes, increasing the severity of the dehydration stresses. Therefore, water that is not bound can freeze and thus, be removed from the cell causing dehydration and ice crystals in the extracellular region. Additionally, bound water inside the cell will freeze if temperatures are low enough. As progressively lower temperatures are achieved more bound water freezes.

The genetic background of an inbred line or a hybrid plays an important role in freezing tolerance. The maternal parent is much more influential than the paternal parent (Rossman, 1949a). Rossman speculated that the stronger maternal influence is related to endosperm characteristics rather than to the pericarp. However, Woltz (2003) found that endosperm characteristics, such as sugary or starchy endosperm, do not influence viability or vigor after freezing.

Husk protection is also a maternal trait, but its importance in preventing freezing injury is not fully understood. It is known that the husks act as an insulator and “buffers” temperature changes (Rossman, 1949a; Fick 1989). However, there is not an apparent relationship between husk insulation and the extent of freezing injury (Rossman, 1949a). The husk is the most important maternal tissue impacting ice formation in the ear. Fick (1989) hypothesized that the husks may be a source

for ice nucleation. In his study, corn ears with husks completely intact responded similarly to corn ears surrounded by husks with the vascular connections severed where the husks attach to the ear. Therefore, ice propagation did not travel from a nucleation source in the husks to the ear via the vascular tissue. If the husks are the ice nucleation point, as proposed by other researchers, ice propagation would have to travel through the layers of husk to the ear. Fick found that the extent of the damage in ears frozen with husks intact is the same as ears still attached to the mother plant and that removing the husks decreases seed vigor (Fick, 1989). Woltz (2003) confirmed that detachment of the ear from the plant does not increase the severity of frost damage as long as the husks are intact. Although genotype is known to influence the effect of frost, the cause of this difference is not explained simply by endosperm composition or husk phenotype.

The physical, mechanical, biochemical, and physiological changes associated with freezing are well established. It is also known that freezing can lead to decreased vigor, decreased viability, and increased electrolyte leakage. However, there is not a good indicator of the extent of fall frost damage in a seed lot, or an established protocol to identify frost damaged seed in a timely fashion to facilitate prompt seed lot marketing decisions.

Several seed quality tests stress seeds before and/or during germination to determine seed vigor. The cold test and all its alternative protocols are probably the most commonly used vigor tests for seed corn in the Midwest. All cold tests have three common factors: low temperature, usually around 10 °C; excessive moisture, enough for exposing the embryo to nearly anaerobic conditions; and non-sterile soil as a source of soil-pathogen inoculation (Burris and Navratil, 1979). Cold tests are used to predict early field emergence; however, the protocols and degree of correlation with field emergence vary greatly (Burris and Navratil, 1979). The saturated cold test is considered the most stressful cold test and is the most common test used by seed companies (Gutormsen, 1996). In the saturated cold test the embryo is placed down into the soil and water which leads to respiratory stress due to limited gas exchange (Hoegemeyer and Gutormsen, 2000). At the

same time CO<sub>2</sub>, ethanol, and acetaldehyde accumulate in the seed and surrounding media (Martin et al., 1988; Hoegemeyer and Gutormsen, 2000). In a later study, Martin et al. (1991) suggested that CO<sub>2</sub> inhibits germination in corn seeds subjected to soaking in water prior to germination. They proposed that, although the saturated cold test is more time consuming than the soak test, it has a higher correlation to field emergence than the soak test (Martin et al., 1988). Martin et al. (1988) speculated that excessive dehydration of the seed during drying may result in the differences between the saturated cold test and the soak test. Accelerated aging test is a relatively simple seed vigor test, however it is not widely used in seed corn. Originally the accelerated aging test was developed to predict storability of a seed lot (Delouche and Baskin, 1973). However, the results from this test correlate well to field emergence (Egli and TeKrony, 1996). There are three stress variables in the accelerated aging test: increased temperature compared to storage; nearly 100% relative humidity; and duration seeds are exposed to aging. These conditions lead to increased respiration and metabolism, during which low vigor seeds deteriorate rapidly (McDonald, 1998).

The degree of seed freezing injury in seed lots can be indirectly measured with the electrical conductivity test. However, this test is used primarily for research purposes in maize seed and it is not commonly used in industry. The electrical conductivity measures the amount of solutes which leak from seeds when they imbibe water prior to germination (McDonald, 1998). The higher the electrical conductivity values, the more the cells in the seed leaked upon rehydration, and the lower the vigor of the seed lot. Electrical conductivity values are lower in the treatments where frost damage is less severe. For example, seeds frozen with the husks removed had higher electrical conductivity values than seeds frozen with the husks intact (Fick, 1989).

Few studies have evaluated multiple seed quality tests (SC, Soak, and AA) for determining frost damage in maize seed. Fick (1989) reported that the results of the standard germination test were not significantly different from those of the sterile cold test for frozen seed lots. Woltz (2003) found that frost damaged seed lots

responded differently when tested in the cold test and the accelerated aging test. In these experiments, the standard germination and accelerated aging tests were significantly different for seeds frozen at early maturities, while the cold test results were not. Moreover, the electrical conductivity test results provided little additional information for identifying or measuring frost damage (Woltz, 2003). These experiments provided an insightful view of the relationship among some seed quality tests and frost damage in seeds. However, a detailed comparison of all these tests and their changes over storage time has not been conducted.

The objectives of this study are to determine which seed quality test is best for identifying frost damaged seed lots and how soon frost damage becomes apparent as measured by seed quality tests in maize.

## **Materials and Methods**

### **Seed Production and Frost Treatment**

The field experiments were planted in 2003 and 2004 near Ames, Iowa. In 2003, an isolation plot was planted at the agronomy research farm west of Ames following a randomized complete block design (RCBD) with 5 blocks. In 2004, two locations were planted at the agronomy research farm. The design at each location was a RCBD, one with 3 blocks and the other with 4 blocks. The planting dates, growing conditions, and harvest periods for each location are shown in Table 1. Each experimental unit was a seed lot harvested from a field plot which had been assigned a block, genotype, and moisture content at harvest. Once harvested, the ears from each plot were divided in two groups and randomly assigned either the frost treatment or designated a control sample. Frost treatment, therefore, led to a split-plot design.

Field plots were seeded at 61,750 plants/hectare with a final stand between 49,000 and 54,000 plants/hectare. Hybrid seed was produced in isolation by open pollination. The female inbreds used in the cross were B73 and Mo17. A common male parent, IRF311, was selected because of its synchrony of flowering with both female parents. B73 was developed at Iowa State University by a team of university

and USDA breeders. This inbred is considered relatively cold-tolerant at the seedling stage (Mock and McNeill, 1979). Mo17 was developed at the University of Missouri and is considered relatively cold-sensitive at the seedling stage. The pollinator IRF311 pollinator was developed at Iowa State University and was obtained from the Iowa Research Foundation.

Field plots were harvested at 500-550, 400-450, or 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw. The final harvest was done after the seed reached physiological maturity. The ears were harvested from the field plots with the husk and shank intact. All ears were placed in 10°C for 2-4 h to prechill the ears and ensure that the freezing rates for all harvests were similar. In 2003, control ears remained in the 10°C chamber while the frost treated ears were placed in a Conviron growth chamber (Controlled Environment Limited, Winnipeg, Manitoba, Canada) with a 24 h programmed freeze cycle. In 2004, control ears were husked and dried following the 2-4 h prechill to avoid the low level of damage observed in all control samples in 2003. An artificial frost cycle (Figure 1) was developed to mimic typical early fall frost temperature patterns in Iowa (Dr. Elwin Taylor, personal communication). The entire cycle lasted 24 h, beginning and ending at 10°C. It included a total of 9 h below 0°C with 2 h of this time at -5°C. This temperature cycle was severe enough to be considered a hard killing frost.

In 2003, all ears were placed in the 10°C chamber for an additional 24 h to thaw before being husked. In 2004, frost treated ears were husked immediately following the frost cycle. After ears were husked, they were placed in small-scale laboratory dryers as described by Navratil and Burris (1982) and dried with forced air at temperatures below 35°C until the seed was at 120 g H<sub>2</sub>O kg<sup>-1</sup> fw. Dried ears were shelled (Custom Seed Equipment, Altoona, Iowa) and seed was stored in 10°C and 50% RH until the seed quality tests were conducted. Seed testing was conducted at 0, 1.5, 3, 4.5, and 6 mo after the beginning of storage.

The 12 field blocks were used as replications (or blocks) in the lab. Each field block had 12 treatment combinations of female\*moisture\*frost treatment for a total of 144 seed lots. One hundred seeds from each seed lot were used for each seed



quality test. Seed testing was repeated in time at 0, 1.5, 3, 4.5 and 6 mo after the beginning of storage.

#### Seed Quality Tests

Standard germination tests were done according to AOSA Rules for Testing Seeds (2004). Each replication consisted of 100 seeds per seed lot and treatment. In 2003, rolled towel media (Anchor, Hudson, WI) was used for the standard germination test. In 2004, crepe cellulose paper media (Kimberly Clark, Neenah, WI) was used for the standard germination test instead of rolled towels. Both methods are allowed in the AOSA Rules for Testing Seeds (2004). The final evaluation was made at 7 d according to AOSA Rules for Testing Seeds (2004). Standard germination tests were conducted at constant temperature (25°C) and alternating light (4 h on/ 4 h off) for a total of 12 h of light per day.

Saturated cold tests were conducted as described by Martin et al. (1988) with the following changes. After 7 d at 10°C, the trays were moved to 25°C and alternating light for 4 d before being evaluated. Only 100 seeds were used for each seed lot. Seedlings were evaluated according to the AOSA Rules for Testing Seeds (2004).

Accelerated aging tests were done according to the Seed Vigor Testing Handbook (AOSA, 2002) with the following exceptions. Samples of 100 seeds were placed on a mesh screen above 40 ml water inside a plastic box (approximately 100% RH) and aged at 43°C for 72 hours. After the aging process seeds were placed in a crepe cellulose paper (Kimberly Clark, Neenah, WI) germination test for 7 days. Seedlings were evaluated according to the AOSA Rules for Testing Seeds (2004).

The soak tests were conducted as cited in Martin et al. (1991) with the following changes. Two reps of 50 seeds were placed in 9 oz plastic cups with 150 ml deionized distilled water for 48 h at room temperature (20-25 °C). Water was drained and the two reps were combined, for a total of 100 seeds per seed lot, and germinated on crepe cellulose paper (Kimberly Clark, Neenah, WI) for 7 days. Seedlings were evaluated according to the AOSA Rules for Testing Seeds (2004).

### Field Emergence

One hundred seeds from each seed lot were planted in completely random design growouts in the spring. There were two planting dates for each year; the first was early (poor field conditions), and the second was later (average to good growing conditions). A summary of the field emergence conditions is shown in Table 2. Number of seedlings was counted at approximately the V2 stage.

### Statistical Analysis

The analysis of variance (ANOVA) and mean comparisons were done using PROC MIXED in Statistical Analysis Software (SAS Institute Inc., Cary, NC). Analysis was done by time with block nested within location as the random effect. Fixed effects were year, and all combinations of female, moisture, and frost treatment. Regressions were done using PROC REG in SAS. T-test values to compare the slopes of the regression lines were calculated based on the method outlined in Biostatistical Analysis (Zar, 1996).

## **Results and Discussion**

The ANOVA for the standard germination and saturated cold test at each time interval is shown in Table 3. Patterns for each test were consistent over time. In the standard germination test, the three way interaction between female, moisture and frost treatment was not significant. The female by frost treatment interaction was not significant other than at 0 mo. Environment was significant at all testing times other than 0 mo.

The other main effects, female, moisture and frost treatment, as well as the female by moisture and the moisture by frost treatment interactions were significant at all times. In the saturated cold test the following interactions were not significant: female by moisture, female by frost treatment, and female by moisture by frost treatment. The female main effect was significant only at 1.5 and 3 mo. All other factors in the model were significant across all testing periods.

The effect of environment on seed quality results can be broken down into year effect and location within year effects. The effect of year was significant

( $P \leq 0.05$ ), while the two locations planted within the same year were not significantly different (data not shown). These results were true for all tests and testing periods in this study. Table 1 is a summary of the seed production environments. In 2003, conditions were very dry and warm during grain fill which significantly impacted seed quality across the Midwest (Goggi et al., 2006). However in 2004, there was adequate precipitation and very few stress days. Although the planting dates between the two fields in 2004 were two weeks apart, the growing conditions were very similar (Table 1). The total precipitation and number of stress degree days between planting and harvesting were similar, helping to explain why the year effect was significant while the location effect was not.

Testing time was significant ( $P < 0.05$ ); however, seed quality did not decrease significantly over time. Since seed was stored in a cold room, the decline in seed quality may have been too slow to measure within the duration of these experiments. This decline in seed quality could also occur after the first 6 mo of storage. Seed samples were also tested at 12 or 18 mo from the beginning of storage. These results indicated that the seed quality of some samples had changed, while in most it remained the same (data not shown). Because there was no distinct decline in seed quality over time and testing time did not have a significant interaction with female and moisture, the seed quality test data for female and moisture were averaged over the five testing periods. Preliminary data analysis showed that the percentage of abnormal seedlings did not change in response to frost (data not shown). Thus, data analysis was conducted only on the percentage of normal seedlings. In a previous study, the number of dead seeds in the TZ test increased in response to frost, but the number of low vigor seeds did not change (DeVries and Goggi, 2006).

The main factors in this study were the effect of frost on two genotypes with different female parents and moisture content at harvest. The mean germination percentage between the frost treatments (control and frost treated seed lots) for all the seed quality tests are presented in Tables 4-7. Mean separations were calculated for the mean difference between the germination percentage of the seed

from the control samples minus the germination percentage of the frost treated seed. Thus, germination percentages in Table 4 represent the change in germination percentage of the seed lots due to the frost treatment. Because the female by moisture interaction, female by frost treatment interaction, and moisture by frost treatment interactions were significant for several of the tests and testing times, data were analyzed within female and moisture content. All data analyses are presented by female and moisture content for all tests, even though the female by moisture interaction was not significant for the saturated cold test at any of the testing times.

Table 4 contains the mean standard germination (or warm germination, WG) percentage for control and frost damaged seed lots, and the mean difference in germination percentage between these frost treatments, expressed by female parent and by seed moisture content at harvest. The artificial frost treatment significantly decreased germination percentage of the hybrids from both females at all moisture contents except for the hybrid from Mo17 at physiological maturity (300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw). The hybrid from B73 responded similarly to frost when at 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw and 400-450 g H<sub>2</sub>O kg<sup>-1</sup> fw. The impact of frost at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw was significantly greater for both hybrids. On the average, the standard germination percentage in frost damaged samples was 30% lower than the control samples. The hybrid from Mo17 at physiological maturity was not impacted by frost, but was significantly damaged when seeds were at 400-450 g H<sub>2</sub>O kg<sup>-1</sup> fw and 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw. Thus, the hybrids from B73 and Mo17 responded differently to frost.

Table 5 presents the results from the accelerated aging test (AA). For the hybrid with B73 as the female parent, the mean differences between control and frosted samples in the AA test were not significantly different for seeds harvested at 300-350 and 400-450 g H<sub>2</sub>O kg<sup>-1</sup> fw, but frost damage was significantly more severe for seeds harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw. The mean differences between control and frosted samples in the AA test were not significantly different for the Mo17 hybrid seed harvested at 400-450 and 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw, while samples frosted at physiological maturity had no damage. The response to frost in the two

hybrids was not significantly different prior to physiological maturity but, once seeds were mature, the Mo17 hybrid was more tolerant to frost than the B73 hybrid.

The results in the saturated cold (SC) and soak tests were similar for both hybrids and the three moisture contents. Results for these two tests are shown in tables 6 (SC) and 7 (soak). The SC and the soak test percentages for the B73 hybrid decreased significantly at all moisture contents. The mean differences between control and frosted samples harvested at 300-350 and 400-450 g H<sub>2</sub>O kg<sup>-1</sup> fw were approximately 10-12%. The mean difference from the early harvest, 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw, was approximately 25%. The SC and soak tests percentages for Mo17 also decreased approximately 12-15% at 400-450 g H<sub>2</sub>O kg<sup>-1</sup> fw and 25% for samples harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw. At physiological maturity the decrease in the SC and soak tests for the Mo17 hybrid was approximately 5%; however, this difference was not significant ( $P \leq 0.05$ ). The vigor of the Mo17 hybrid at physiological maturity was, once again, not significantly affected by frost (Tables 6 and 7). When hybrids harvested at 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw moisture content were compared to each other, the effect of female parent was not significant.

The germination percentages of control and frost treated samples of the B73 hybrid are fairly consistent across all seed quality tests (Tables 4-7). However, the germination percentages of control and frost treated samples of the Mo17 hybrid are higher in the SC and soak tests than in the WG and AA tests. These results are counterintuitive, since the WG percentage is usually higher than the vigor tests. The WG measures germination under ideal growing conditions while vigor tests measure germination under stressful environments. However, the warm conditions of the WG and AA tests also can provide ideal growing conditions for fungal pathogens. These pathogens, if prevalent, can reduce the germination percentage of the seed lot. The stressful growing environments of the SC and soak tests can hinder the proliferation of these pathogens. This reduction in pathogen pressure could explain the higher SC and soak germination percentage observed in these seed lots.

When seeds are at physiological maturity, the hybrid with Mo17 as the female parent shows tolerance to frost in the standard germination and accelerated aging

tests, while the hybrid with B73 as the female parent is damaged by frost. The frost-induced decrease in the SC and soak tests percentages at physiological maturity are 10% for the B73 hybrid and 5% for the Mo17 hybrid, approximately. This difference in germination between control and frost treatment is significant for B73 hybrid but not for Mo17 hybrid. These results indicate that the Mo17 hybrid is more tolerant to frost at physiological maturity than the B73 hybrid. Previous studies found that, at the vegetative stage, B73 is cold tolerant (Stewart et al., 1990). However, recent studies show that these two inbreds behave differently at the reproductive and vegetative stages. Jorgensen et al. (1992) found that Mo17 was more heat tolerant at the vegetative stage than B73. However, Commurri and Jones (2001) found that high temperature significantly affected seed set, development, and the number of endosperm cells in Mo17, while B73 was unaffected. Therefore, they concluded that B73 is more tolerant to high temperatures than Mo17 at the reproductive and seed development stages. Prior to physiological maturity, the seed quality of the two hybrids included in our study is affected similarly by the frost event. Other researchers have shown a strong genetic component associated with seed quality as estimated by accelerated aging (Woltz and TeKrony, 2001; Munamava et al., 2004), saturated cold (Munamava et al., 2004) and soak tests (Martin et al., 1991; Cerwick et al., 1995; Munamava et al., 2004).

Seed moisture content at harvest is important for determining seed quality and the effect of frost. Both hybrids show the most severe damage at the earliest harvest, 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw. As seeds matured, the detrimental effect of frost decreased in both hybrids. For the hybrid with B73 as the female parent, the AA and SC results from control samples harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw were lower than the standard germination test for the same samples. These results lead to smaller percentage differences for seed samples of the B73 hybrid harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw in the AA and SC tests. The standard germination of the Mo17 hybrid decreased from 90.4% in seed lots harvested at physiological maturity to 69.8% in seed lots harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw (Table 4). These seed lots were also less vigorous as estimated by the AA, SC, and soak tests (Tables 5-7). The frost-

damaged seeds from both hybrids had similar germination percentages before and after aging and in the SC test. The lower vigor of control samples of the B73 hybrid harvested at high moisture contents did not change the effect of frost, it simply made the difference between control and frost treated samples smaller. The decrease in vigor in control samples of both hybrids may be associated with premature harvest and drying. Cordova-Tellez and Burris (2002) found that at high moisture content (500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw) some genotypes were sensitive to premature drying even when proper techniques were used. The current study supports this conclusion, and suggests that both B73xIRF311 and Mo17xIRF311 hybrids may be sensitive to drying when harvested above 500 g H<sub>2</sub>O kg<sup>-1</sup> fw.

#### Regressions to field emergence

To determine which seed quality test best predicts field emergence of frost damaged seed, the lab tests results were independently regressed to both early and late field emergence. Only data from frost damaged seed lots were used to calculate the regressions, however the non-frosted seed lots also were included in the graphs and followed along the same linear regression (Figures 2-13). The regressions of laboratory tests to early field emergence were very weak, and the adjusted  $r^2$  values ranged from 0.13 to 0.32 (Table 8). The ideal slope for a regression between a lab test and field emergence would be 1.0, implying that the germination percentage in the laboratory test was equal to field emergence. The slopes of these regressions (0.48 to 0.62), although significant, indicated that the lab tests were overestimating early field emergence, or that field conditions early in the spring were too variable for a strong relationship between field emergence and laboratory test results to be obtained. TeKrony and Egli (1977) also found that regressions between laboratory tests and early field emergence are very poor. In their study, cold test and accelerated aging did not predict emergence in poor field conditions in soybeans. In a later study (Woltz and TeKrony, 2001), found similar results in corn. Because of the variation in the field conditions, the lab test results often overestimate field emergence.

The regressions between the lab tests and the field emergence of the second planting date were much stronger. All regressions and their slopes were significant ( $P < 0.05$ ). Standard germination results after 0, 1.5, and 3 mo of storage were regressed to late field emergence (Figures 2, 3 and 4 respectively). The adjusted  $r^2$  values were between 0.56 and 0.75. The slopes of the regression were 0.64, 0.75, and 0.72 for 0, 1.5, and 3 mo of storage, respectively. The intercepts of these regressions were significant ( $P < 0.05$ ). The slopes and significant y-intercepts indicated that standard germination overestimates the field emergence of frost damaged seed. The standard germination test is designed to give maximum germination under ideal conditions (AOSA, 2002). Field conditions are rarely ideal, therefore, we would expect the standard germination test to overestimate field emergence.

The accelerating aging test at 0 mo was better than the standard germination test for predicting field emergence of frost damaged seed. The adjusted  $r^2$  value was 0.81 and the slope of the regression was 0.88 (Figure 5). The y-intercept was not significant. However, after storing frost damaged seed for 1.5 mo, the adjusted  $r^2$  decreased to 0.62 and there was a significant y-intercept (Figure 6). After 3 mo of storage, the adjusted  $r^2$  was 0.78 and the y-intercept was significant (Figure 7). Therefore, the accelerated aging test was more sensitive than the standard germination test. It is possible that during the early months of storage, some seed recovery and repair occurs. This repair could lead to higher seed quality at 1.5 and 3 mo and thus, seed quality test results that overestimate field emergence. However, directly after harvest and drying, the accelerated aging test is a good measurement of seed vigor and a good predictor of field emergence.

The best seed quality tests for predicting field emergence under average to good field conditions were the saturated cold (SC) and soak tests (Figures 8-13). The adjusted  $r^2$  values of the regressions between late field emergence and the SC and soak tests ranged from 0.77 to 0.86. There were no significant y-intercepts for either of the tests during the first 3 mo of storage. The slopes for both tests indicated a very strong relationship between the SC and soak test and field



emergence. The slopes of the regressions for SC were 0.89, 0.90, and 0.96 at 0, 1.5, and 3 mo of storage, respectively. The slopes for the regressions for soak were 0.95, 0.90, and 0.93 at 0, 1.5, and 3 mo of storage, respectively. By performing t-tests on the slopes of these regression lines (Zar, 1996) it was determined that the slope of the regressions for SC at 0, 1.5 and 3 mo were not significantly different from each other. The slopes of the soak test were also not different, indicating that these two tests were not changing during the first 3 mo of storage. When comparing the slopes of the regressions between these two tests and late field emergence, there were no significant differences between tests. These results confirmed that the two tests are equal in their ability to predict field emergence of frost damaged seed within the first 3 mo of storage.

## Conclusions

Our study confirms previous knowledge that frost damage significantly decreases maize seed quality. Both maternal parent and moisture content at harvest are significant factors in determining the extent of frost damage. The hybrids produced from B73 and Mo17 as maternal parents respond differently to frost during seed maturation. The ability of Mo17 to tolerate a frost event at physiological maturity with no significant decrease in seed quality is very important. This means that when a natural frost event occurs, some female parents can remain in the field without significant damage to seed quality. However, other female parents at the same moisture content may suffer damage that can lower seed quality. The hybrids with a sensitive female parent should be harvested first.

Our study also confirms previous reports that frost damage is less severe as seeds mature and dry down. This highlights the importance of harvesting the more immature seed prior to a frost event. Seed that is more mature can remain in the field because it would be less impacted by the frost. However, this strategy is only successful to a point. At high moisture content ( $\geq 500 \text{ g H}_2\text{O kg}^{-1} \text{ fw}$ ), premature harvest can lower seed vigor even if proper drying, conditioning, and storage conditions are maintained. The control samples for the Mo17 hybrid show

decreased viability and vigor as seeds were harvested at higher moisture content. The control samples for the B73 hybrid harvested at high moisture contents (500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw) were viable in the WG test, but had low vigor in the AA and SC tests. The lower vigor of these seed samples indicates that, at high moisture content, there is a trade-off between damage due to premature harvest and damage due to frost. A mild frost may not cause enough damage to warrant early harvest. However, a severe killing frost similar to our artificial frost event can cause enough damage to justify harvesting early.

When a frost event is severe, viability and vigor are decreased to the same degree. For example, the mean germination percentages of the B73 hybrid harvested at 500-550 g H<sub>2</sub>O kg<sup>-1</sup> fw are 62.8, 63.9, 59.7, and 67.4 for the WG, AA, SC and Soak tests, respectively. This indicates that seeds which are still viable following the frost event are vigorous within the first 6 mo of storage. In severely frosted seed lots, seeds either die or survive the frost. This is consistent with results from the tetrazolium test, where the number of seeds in low and medium vigor categories in a seed lot did not change in response to frost (DeVries and Goggi, 2006). The difference between frost damage seed lots and control samples is number of dead seeds in the seed lot after a frost event.

Laboratory tests did not accurately predict field emergence of frost damaged seed in poor field conditions. WG and AA tests have strong relationships to field emergence in average to good field conditions. However, both tests overestimate field emergence. The SC and Soak tests are the best predictors of field emergence and seed quality of frost damaged seed lots. The results from these two tests are consistent during the first 3 mo of storage, and they are equal to each other in their ability to predict field emergence. If a seed lot is suspected to have frost damage, seed can be tested at any point during the first 3 mo of storage using the SC or Soak tests. The tests will provide an accurate prediction of field emergence. When seed is being sent to winter nurseries shortly after harvest, seed samples can be tested immediately after the seed lots have been dried and shelled. If field data or seed lots are unavailable until the seed is ready for conditioning, samples can be tested

after a period of storage (up to 3 mo according to this study). Management decisions can be based on the strong relationship between the SC and Soak tests and field emergence in average to good field conditions.

Results from these experiments advance our understanding of the importance of seed moisture content and maternal parent on the extent of frost damage in immature maize seeds. Seed companies can make harvesting decisions based on the probability and severity of a fall frost forecast. If the seed lots are frost damaged, common seed quality tests can predict field emergence of these seed lots. These seed quality tests are important tools for making management decisions about hybrid seed production in the event of an early fall frost.

Future research to assess the effect of a mild frost on maturing maize seeds may be beneficial for understanding of the potential damage of such an event. When harvesting high moisture content ( $>500 \text{ g H}_2\text{O kg}^{-1}$ ) maize seed, it is possible to have damage associated with premature harvest in addition to any treatment effect.

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Table 1. Seed production environments.

	Planting Date of Female Parent	Growing conditions (GDD10C, Precipitation (mm), and number of stress days (high temperature above 30°C) from planting date of the female parent to 1 <sup>st</sup> harvest date)†	Harvest Period
1	05/16/2003	1174 GDD, 171 mm precipitation, 45 stress days	09/02/2003-09/25/2003
2	04/28/2004	1138 GDD, 423 mm precipitation, 3 stress days	09/01/2004-09/23/2004
3	05/11/2004	1114 GDD, 431 mm precipitation, 3 stress days	09/06/2004-09/30/2004

† Data obtained from Iowa Environmental Mesonet, Iowa State Ag Climate Network at the Ames location

Table 2. Field Emergence Conditions

Seed Production Year	Planting Date	Field Conditions (GDD10C, Precipitation (mm) and Soil GDD10C) from Planting to Evaluating†	Evaluation Date
2003	04/08/2004	72.8 GDD, 44.4 mm, 55.6 Soil GDD	04/27/2004
	06/02/2004	199.4 GDD, 50.0 mm, 241.1 Soil GDD	06/21/2004
2004	04/18/2005	143.9 GDD, 39.9 mm, 142.8 Soil GDD	05/20/2005
	05/24/2005	153.3 GDD, 51.1 mm, 178.3 Soil GDD	06/10/2005

† Data obtained from Iowa Environmental Mesonet, Iowa State Ag Climate Network at the Ames location



Table 3. Analysis of variance (degrees of freedom (df) and *P* values) for standard germination and saturated cold test after 0, 1.5, 3, 4.5 and 6 months in storage.

Standard Germination Source of Variation	df	Months in storage prior to seed testing				
		0	1.5	3	4.5	6
Environment†	9	NS‡	*	**	*	*
Female	121	***	***	***	***	***
Moisture	121	***	***	***	***	***
Female*Moisture	121	***	***	***	***	***
Frost	121	***	***	***	***	***
Female*Frost	121	*	NS	NS	NS	NS
Moisture*Frost	121	***	***	***	***	***
Female*Moisture*Frost	121	NS	NS	NS	NS	NS
Saturated Cold Test Source of Variation						
Environment†	9	*	*	**	**	*
Female	121	NS	*	*	NS	NS
Moisture	121	***	***	***	***	***
Female*Moisture	121	NS	NS	NS	NS	NS
Frost	121	***	***	***	***	***
Female*Frost	121	NS	NS	NS	NS	NS
Moisture*Frost	121	***	**	***	***	***
Female*Moisture*Frost	121	NS	NS	NS	NS	NS

\*, \*\*, \*\*\* Significant at the 0.05, 0.01 and 0.001 probability level respectively.

†Environments explained in more detail in Table 1.

‡NS Not significant at the 0.05 probability level.

Table 4. Effect of frost treatment on standard germination test for two hybrids harvested at three moisture contents. Female parents of the hybrids are B73 and Mo17 with IRF311 as the common male parent.

Moisture Content (MC) at Harvest	Hybrid with B73 female parent				Hybrid with Mo17 female parent				
	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Female within MC ‡
300-350 g kg <sup>-1</sup> fw §	95.1	84.9	10.2 **	a	90.4	88.9	1.4 NS ¶	a	*
400-450 g kg <sup>-1</sup> fw	95.5	81.7	13.7 ***	a	87.7	74.1	13.5 ***	b	NS
500-550 g kg <sup>-1</sup> fw	93.2	62.8	30.4 ***	b	69.8	46.1	23.7 ***	c	NS

\*, \*\*, \*\*\* Mean difference between control and frost treated seed lots is significant at the 0.05, 0.01 and 0.001 probability level respectively.

† Mean differences within a column with the same letter are not significantly different at the 0.05 probability level.

‡ Mean differences for the two hybrids within moisture content are significantly different at the 0.05 probability level.

§ 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw is physiological maturity

¶ NS, Not significantly different at the 0.05 probability level.

Table 5. Effect of frost treatment on accelerated aging test for two hybrids harvested at three moisture contents. Female parents of the hybrids are B73 and Mo17 with IRF311 as the common male parent.

Moisture Content (MC) at Harvest	Hybrid with B73 female parent				Hybrid with Mo17 female parent				
	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Female within MC‡
300-350 g kg <sup>-1</sup> fw§	92.5	82.2	10.3 **	a	89.0	87.2	1.8 NS¶	a	**
400-450 g kg <sup>-1</sup> fw	91.2	80.9	10.4 **	a	89.7	74.8	15.0 ***	b	NS
500-550 g kg <sup>-1</sup> fw	84.0	63.9	21.8 ***	b	76.5	55.6	20.9 ***	b	NS

\*, \*\*, \*\*\* Mean difference between control and frost treated seed lots is significant at the 0.05, 0.01 and 0.001 probability level respectively.

† Mean differences within a column with the same letter are not significantly different at the 0.05 probability level.

‡ Mean differences for the two hybrids within moisture content are significantly different at the 0.05 probability level.

§ 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw is physiological maturity

¶ NS, Not significantly different at the 0.05 probability level.

Table 6. Effect of frost treatment on saturated cold test for two hybrids harvested at three moisture contents. Female parents of the hybrids are B73 and Mo17 with IRF311 as the common male parent.

Moisture Content (MC) at Harvest	Hybrid with B73 female parent				Hybrid with Mo17 female parent				
	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Female within MC‡
300-350 g kg <sup>-1</sup> fw§	94.2	83.7	10.5 **	A	95.4	91.2	4.2 NS¶	a	NS
400-450 g kg <sup>-1</sup> fw	92.7	80.6	12.2 ***	A	95.0	81.3	13.7 ***	b	NS
500-550 g kg <sup>-1</sup> fw	81.6	59.7	21.8 ***	B	85.8	62.7	23.1 ***	c	NS

\*, \*\*, \*\*\* Mean difference between control and frost treated seed lots is significant at the 0.05, 0.01 and 0.001 probability level respectively.

† Mean differences within a column with the same letter are not significantly different at the 0.05 probability level.

‡ Mean differences for the two hybrids within moisture content are significantly different at the 0.05 probability level.

§ 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw is physiological maturity

¶ NS, Not significantly different at the 0.05 probability level.

Table 7. Effect of frost treatment on soak test for two hybrids harvested at three moisture contents. Female parents of the hybrids are B73 and Mo17 with IRF311 as the common male parent.

Moisture Content (MC) at Harvest	Hybrid with B73 female parent				Hybrid with Mo17 female parent				
	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Control (% Normal Seedlings)	Treatment (% Normal Seedlings)	Difference (Control - Treatment)	MC within Female †	Female within MC‡
300-350 g kg <sup>-1</sup> fw§	95.8	85.1	10.7 **	a	93.8	88.7	5.1 NS¶	a	NS
400-450 g kg <sup>-1</sup> fw	95.6	82.9	12.7 ***	a	93.3	79.3	13.9 ***	b	NS
500-550 g kg <sup>-1</sup> fw	94.2	67.4	26.8 ***	b	86.0	61.3	24.7 ***	c	NS

\*, \*\*, \*\*\* Mean difference between control and frost treated seed lots is significant at the 0.05, 0.01 and 0.001 probability level respectively.

† Mean differences within a column with the same letter are not significantly different at the 0.05 probability level.

‡ Mean differences for the two hybrids within moisture content are significantly different at the 0.05 probability level.

§ 300-350 g H<sub>2</sub>O kg<sup>-1</sup> fw is physiological maturity

¶ NS, Not significantly different at the 0.05 probability level

Table 8. Summary of the simple linear regressions between the seed quality tests (WG, AA, SC, and Soak) at 0 and 1.5 months and early field emergence (FE1).

Regression	Adjusted $r^2$	Error of the Estimate	Slope of the Regression
FE1*WG0	0.3195	17.22	0.62
FE1*WG1.5	0.2362	18.25	0.54
FE1*AA0	0.1804	18.90	0.56
FE1*AA1.5	0.0772	20.06	0.38
FE1*SC0	0.1957	18.72	0.57
FE1*SC1.5	0.1452	19.30	0.49
FE1*SOAK0	0.1295	19.48	0.48
FE1*SOAK1.5	0.1193	19.59	0.44

Figure 1. Artificial frost cycle developed to mimic an autumn severe killing frost in the Midwest. Segment 1 cooling rate is  $-1\text{ }^{\circ}\text{C/h}$  from  $10$  to  $9\text{ }^{\circ}\text{C}$ , Segment 2 cooling rate is  $-1.4\text{ }^{\circ}\text{C/h}$  from  $9$  to  $0\text{ }^{\circ}\text{C}$ , Segment 3 cooling rate is  $-0.83\text{ }^{\circ}\text{C/h}$  from  $0$  to  $-5\text{ }^{\circ}\text{C}$ , Segment 4 is a constant  $-5\text{ }^{\circ}\text{C}$  for 2 hours, Segment 5 thawing rate is  $4.7\text{ }^{\circ}\text{C/h}$  from  $-5$  to  $9\text{ }^{\circ}\text{C}$ , Segment 6 thawing rate is  $1\text{ }^{\circ}\text{C/h}$  from  $9$  to  $10\text{ }^{\circ}\text{C}$ .

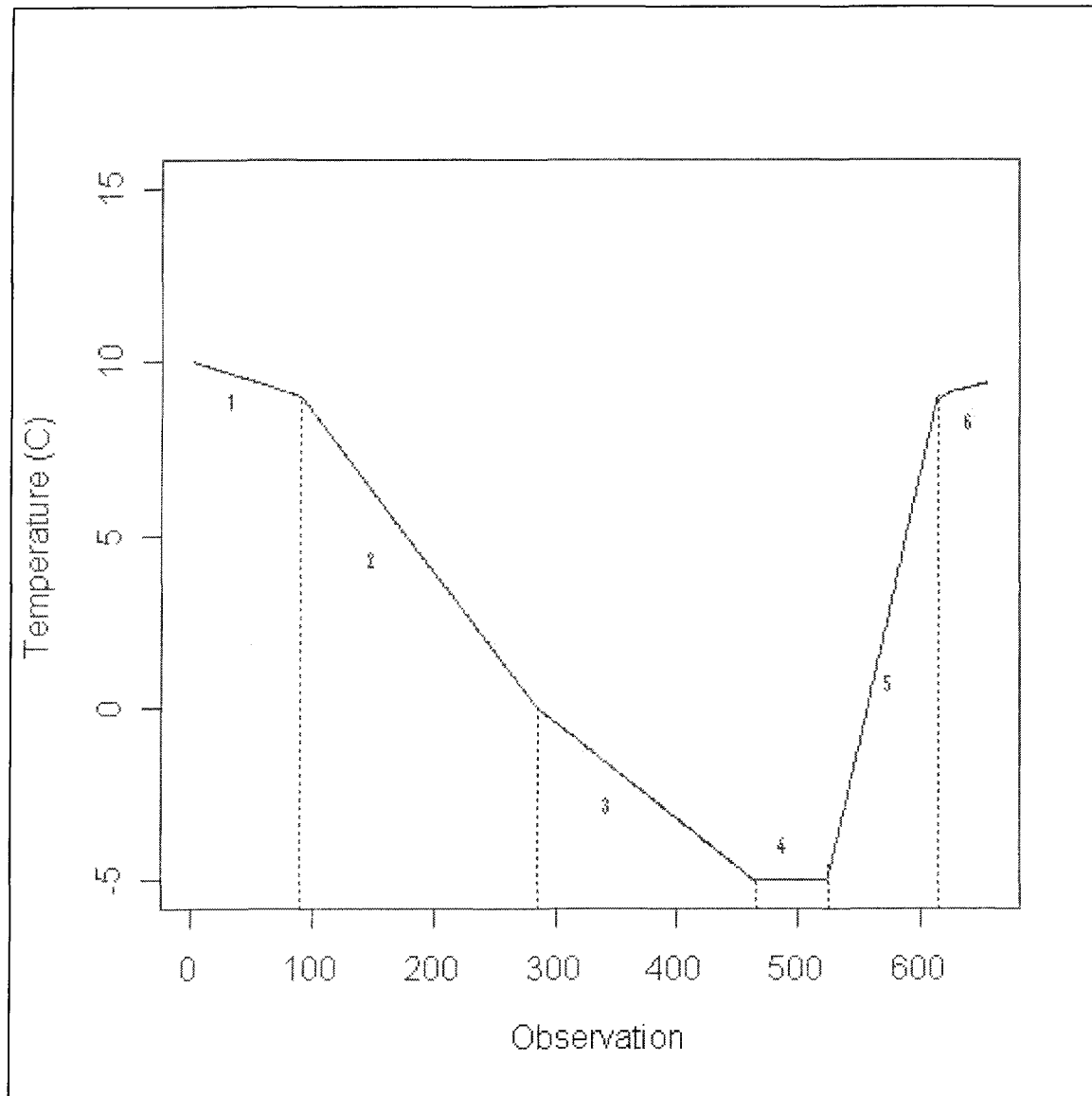


Figure 2. Standard germination at 0 mo (WG0) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between WG0 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

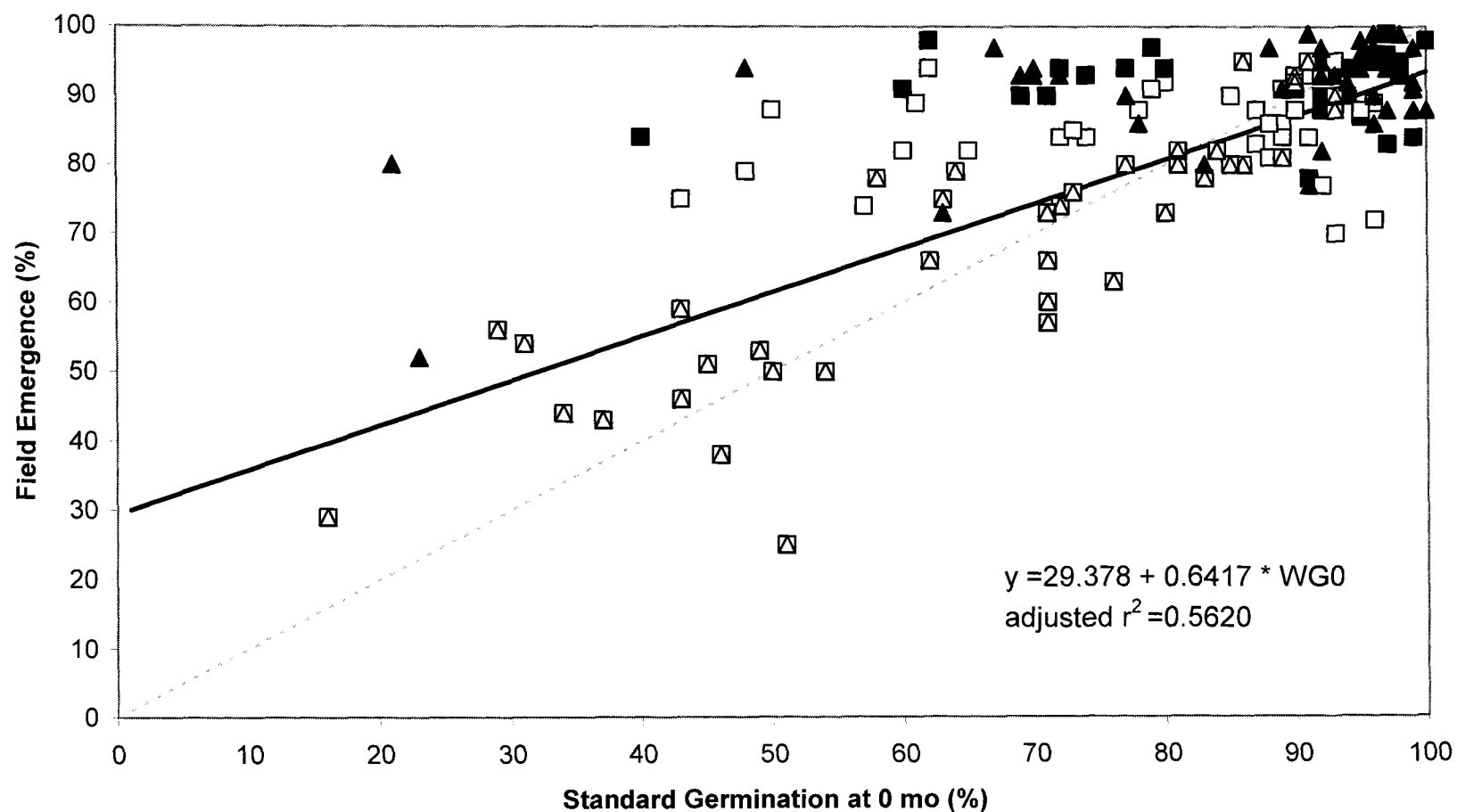




Figure 3. Standard germination at 1.5 mo (WG1) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between WG1 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

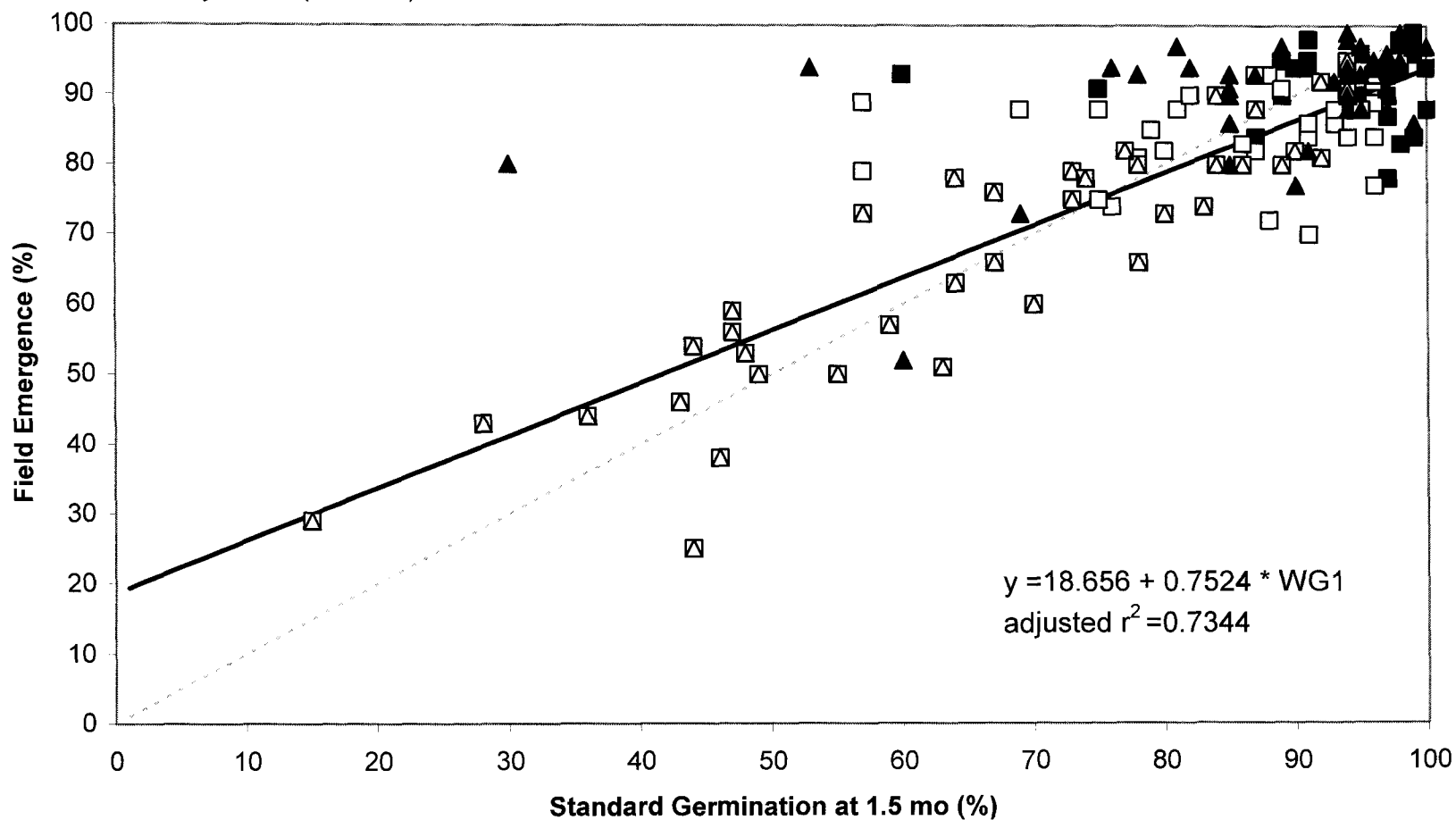


Figure 4. Standard germination at 3 mo (WG3) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between WG3 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

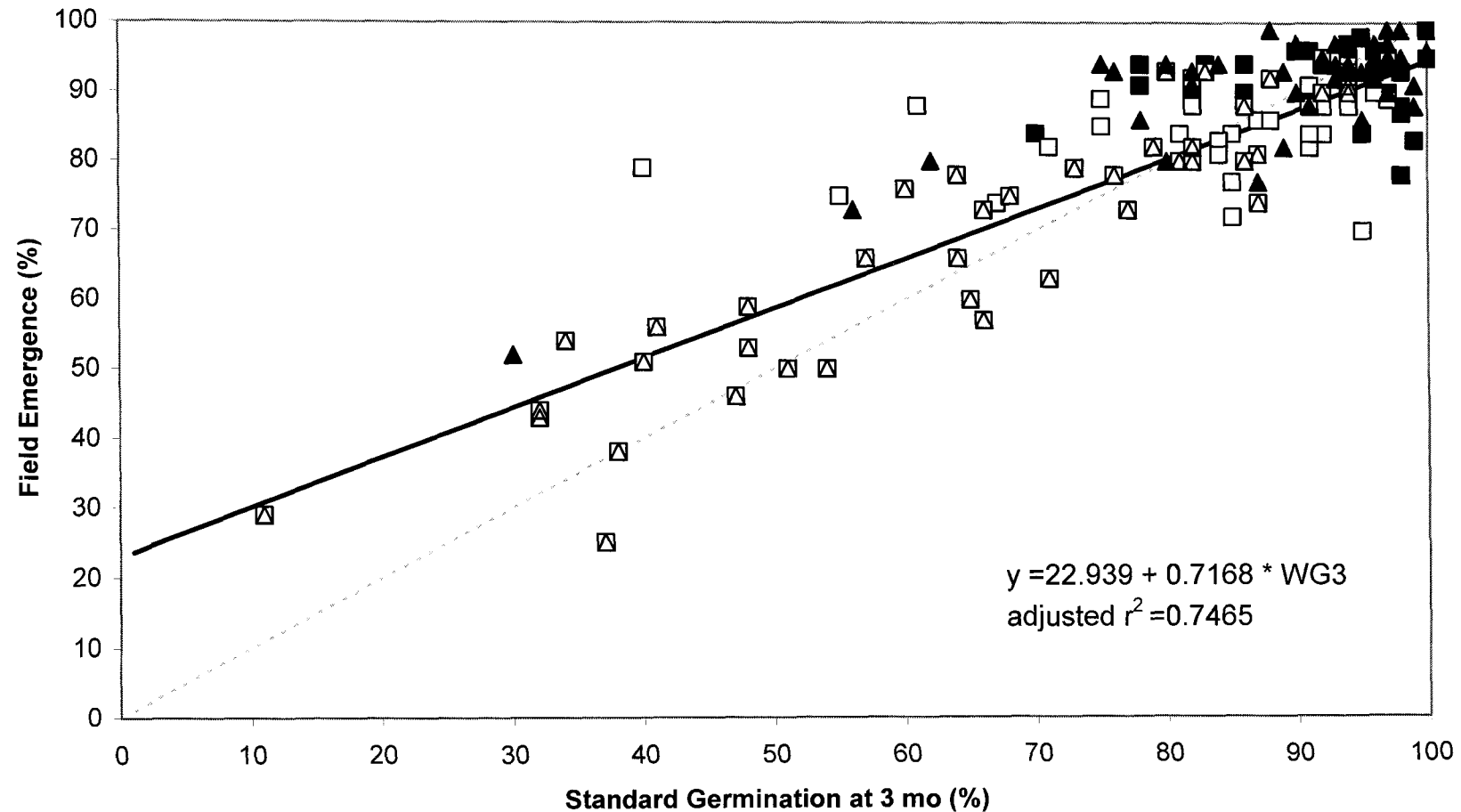


Figure 5. Accelerated aging test at 0 mo (AA0) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between AA0 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

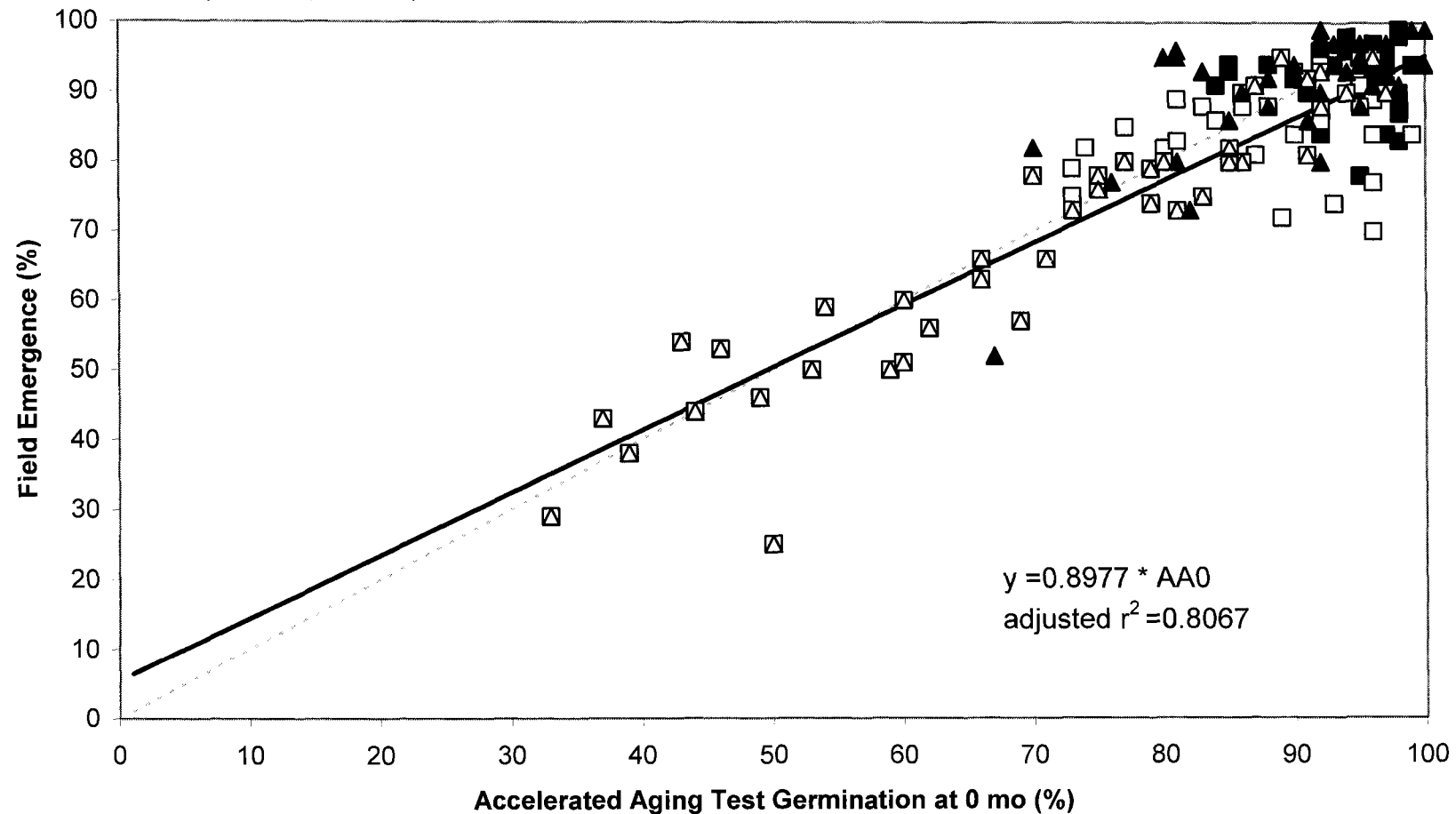


Figure 6. Accelerated aging test at 1.5 mo (AA1) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between AA1 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

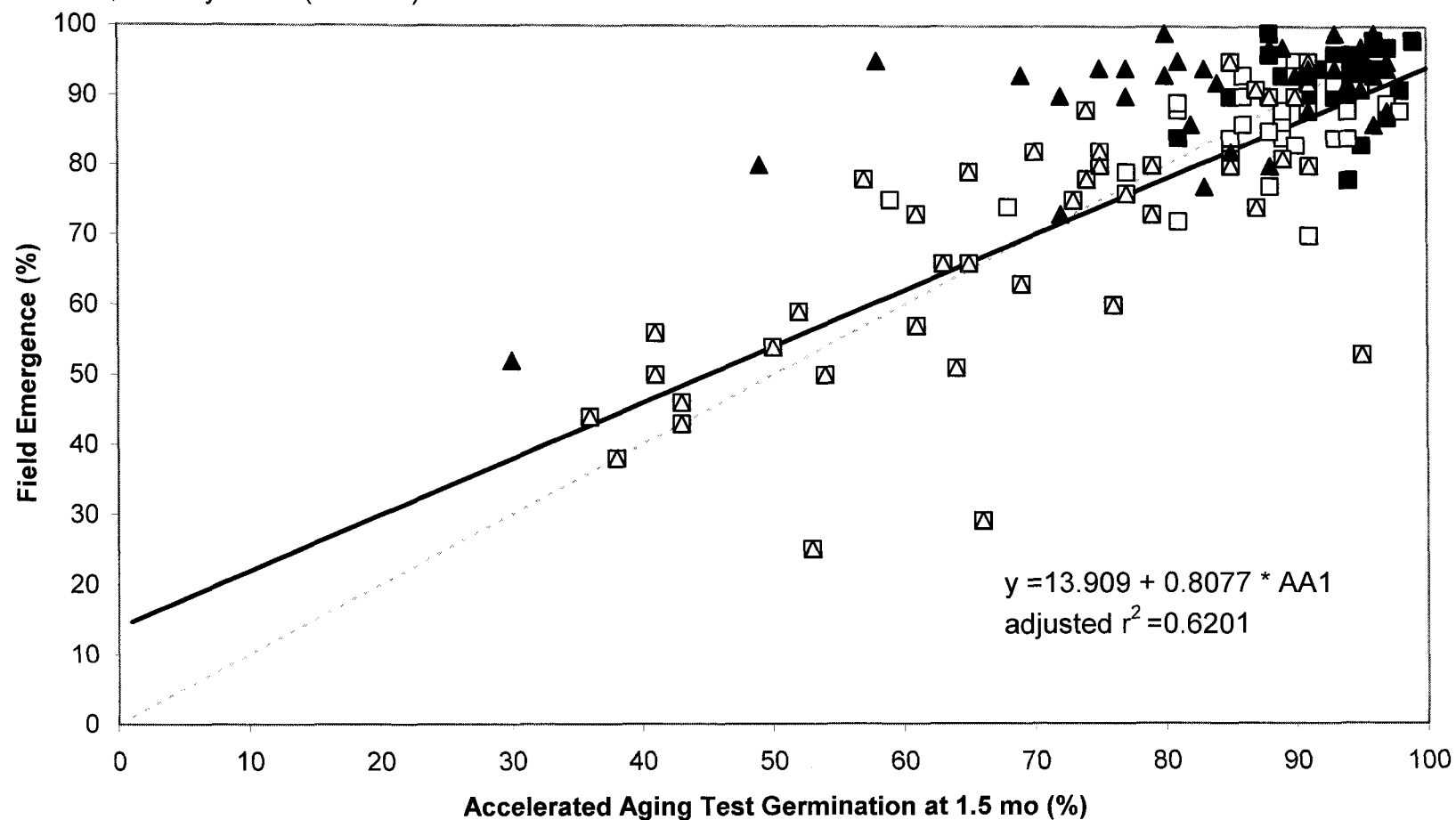


Figure 7. Accelerated aging test at 3 mo (AA3) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between AA3 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

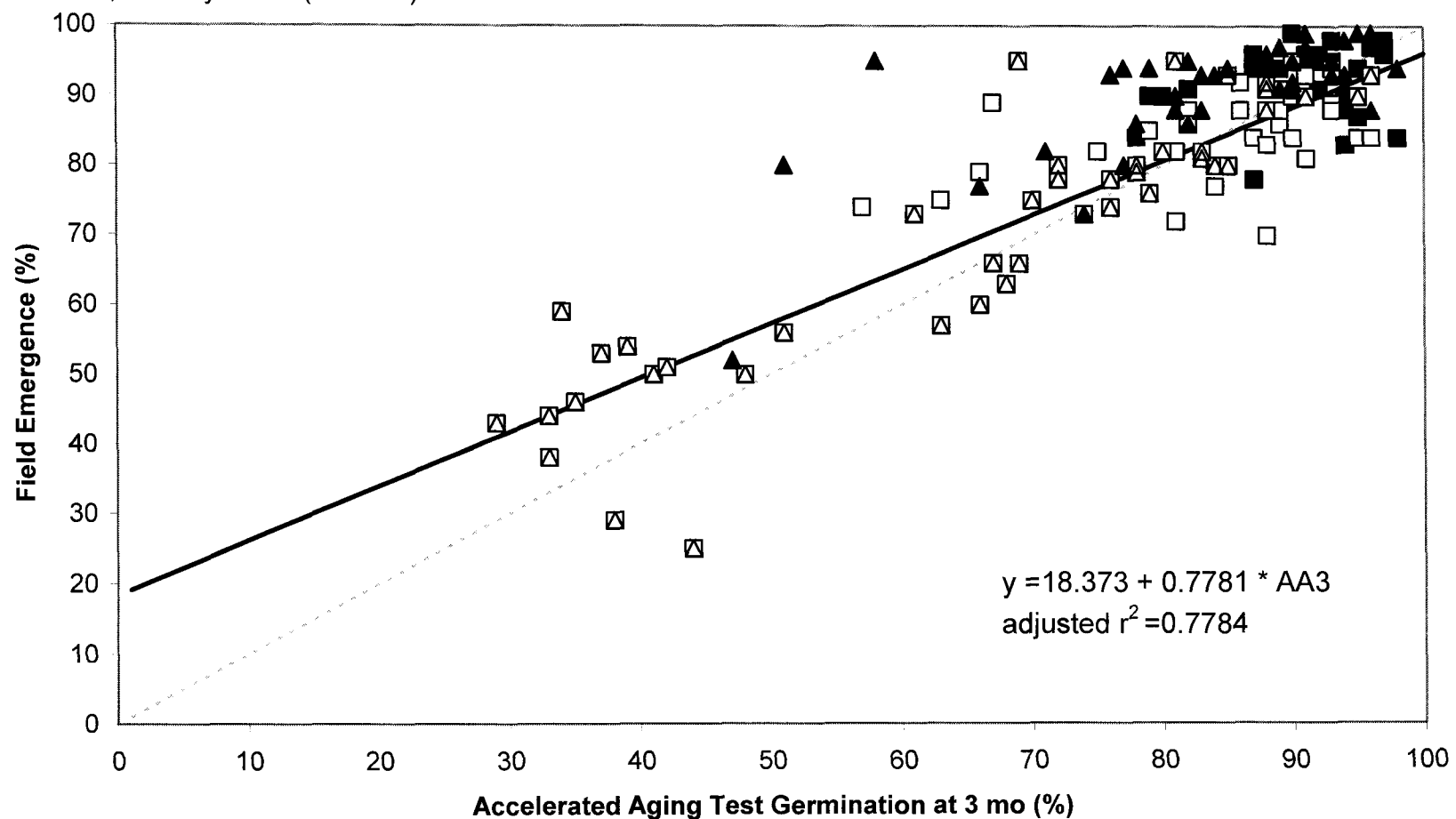


Figure 8. Saturated cold test at 0 mo (SC0) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between SC0 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

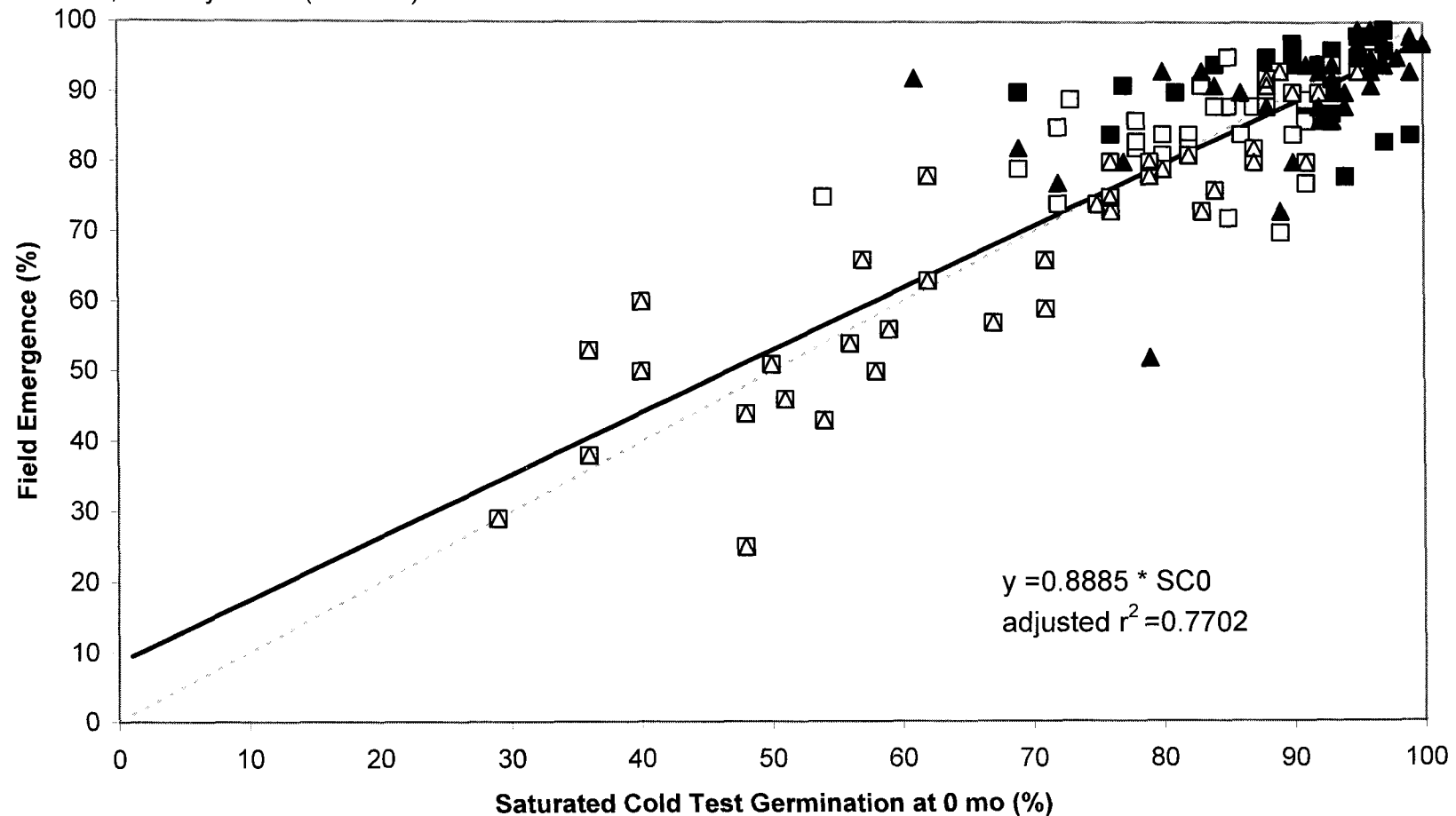


Figure 9. Saturated cold test at 1.5 mo (SC1) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between SC1 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

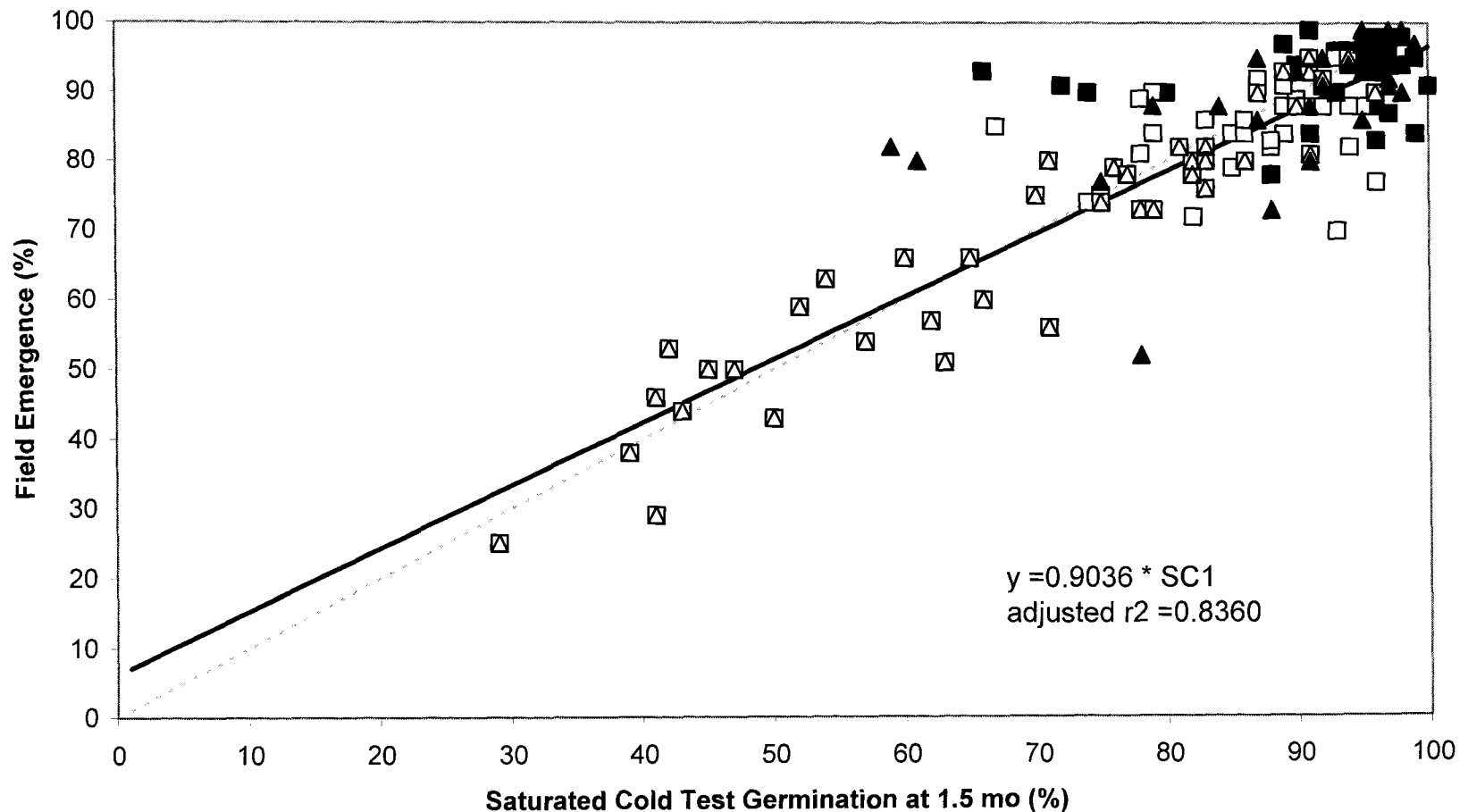


Figure 10. Saturated cold test at 3 mo (SC3) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between SC3 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

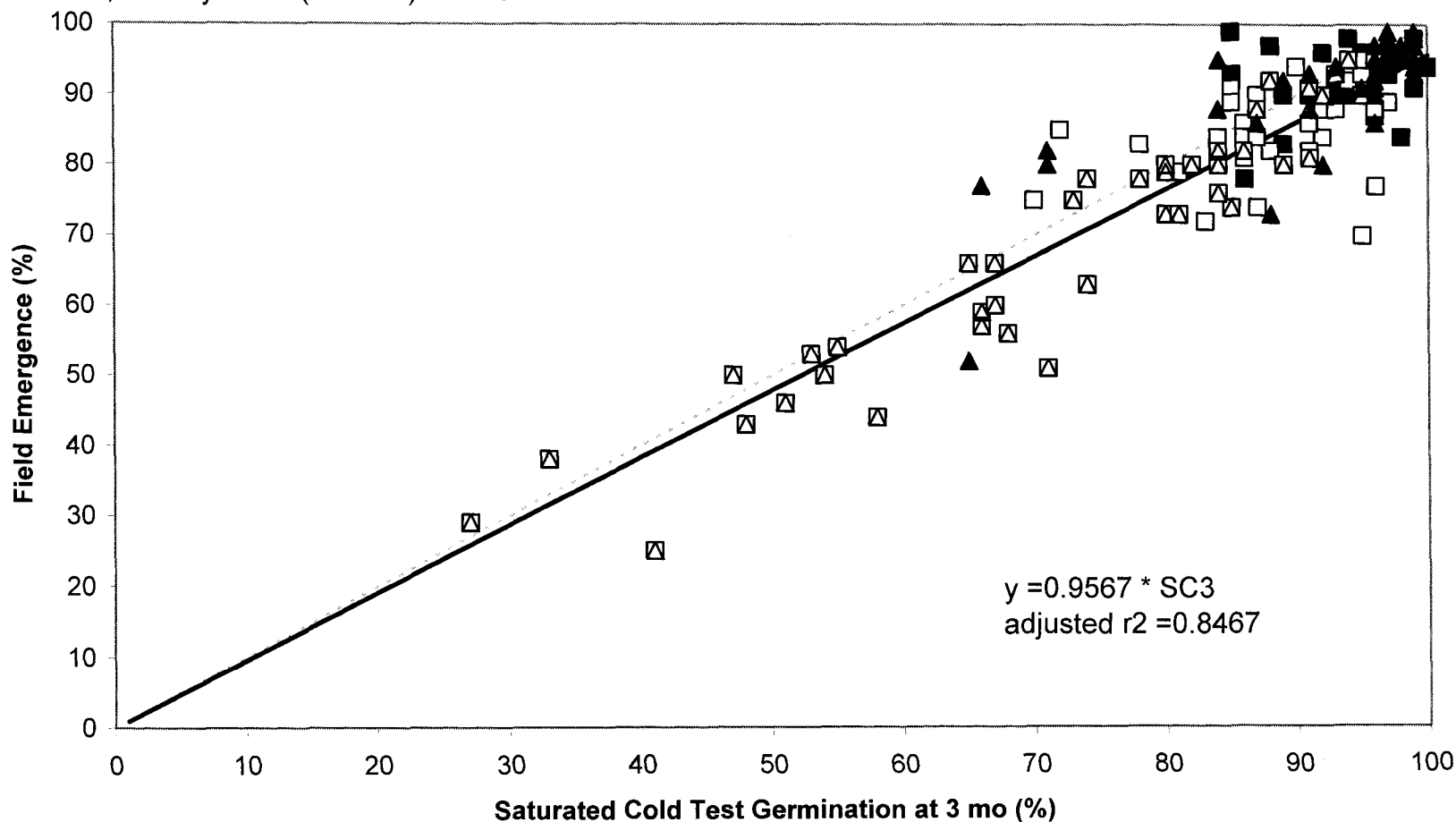




Figure 11. Soak test at 0 mo (Soak0) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between Soak0 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

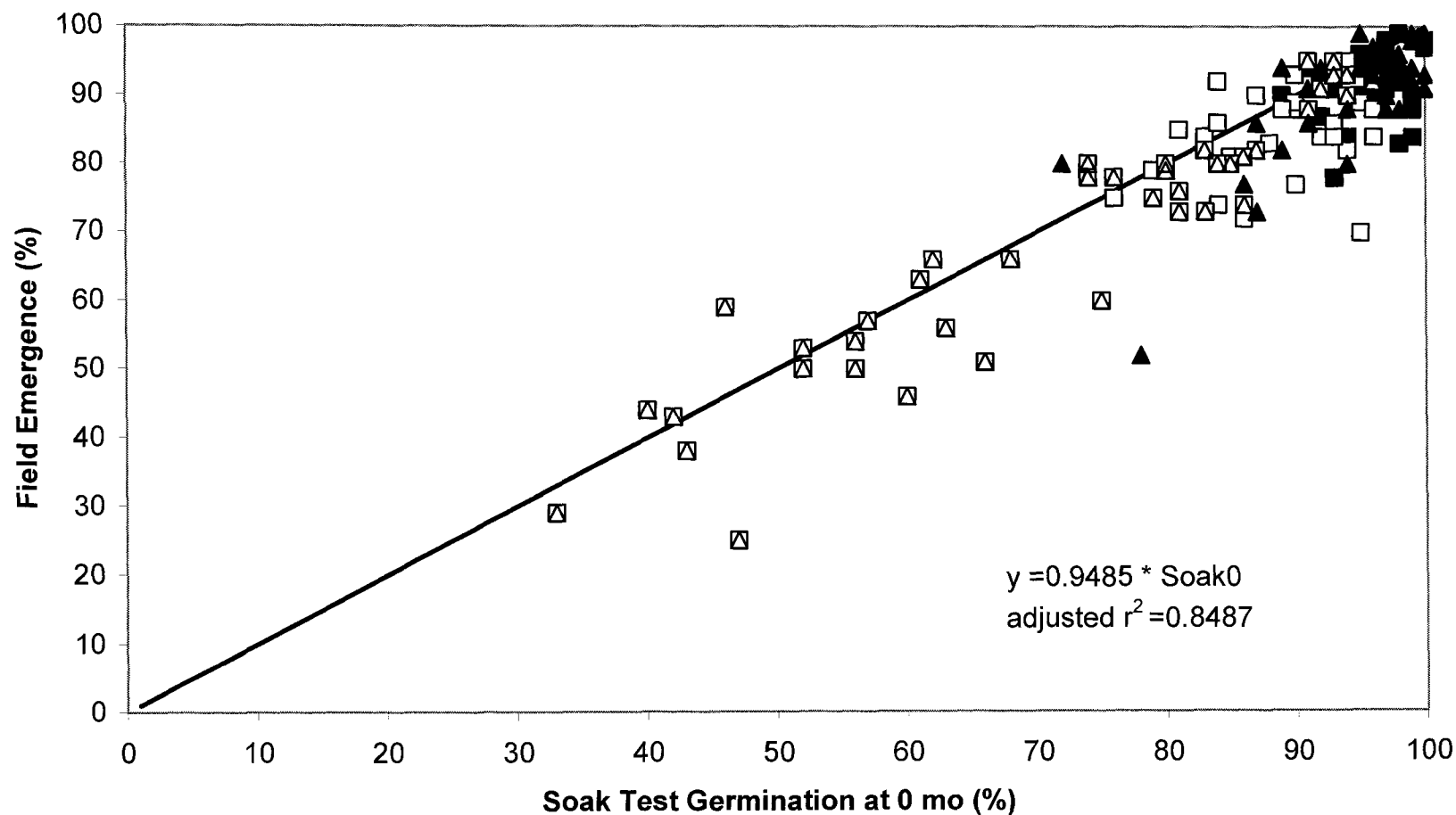


Figure 12. Soak test at 1.5 mo (Soak1) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between Soak1 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible

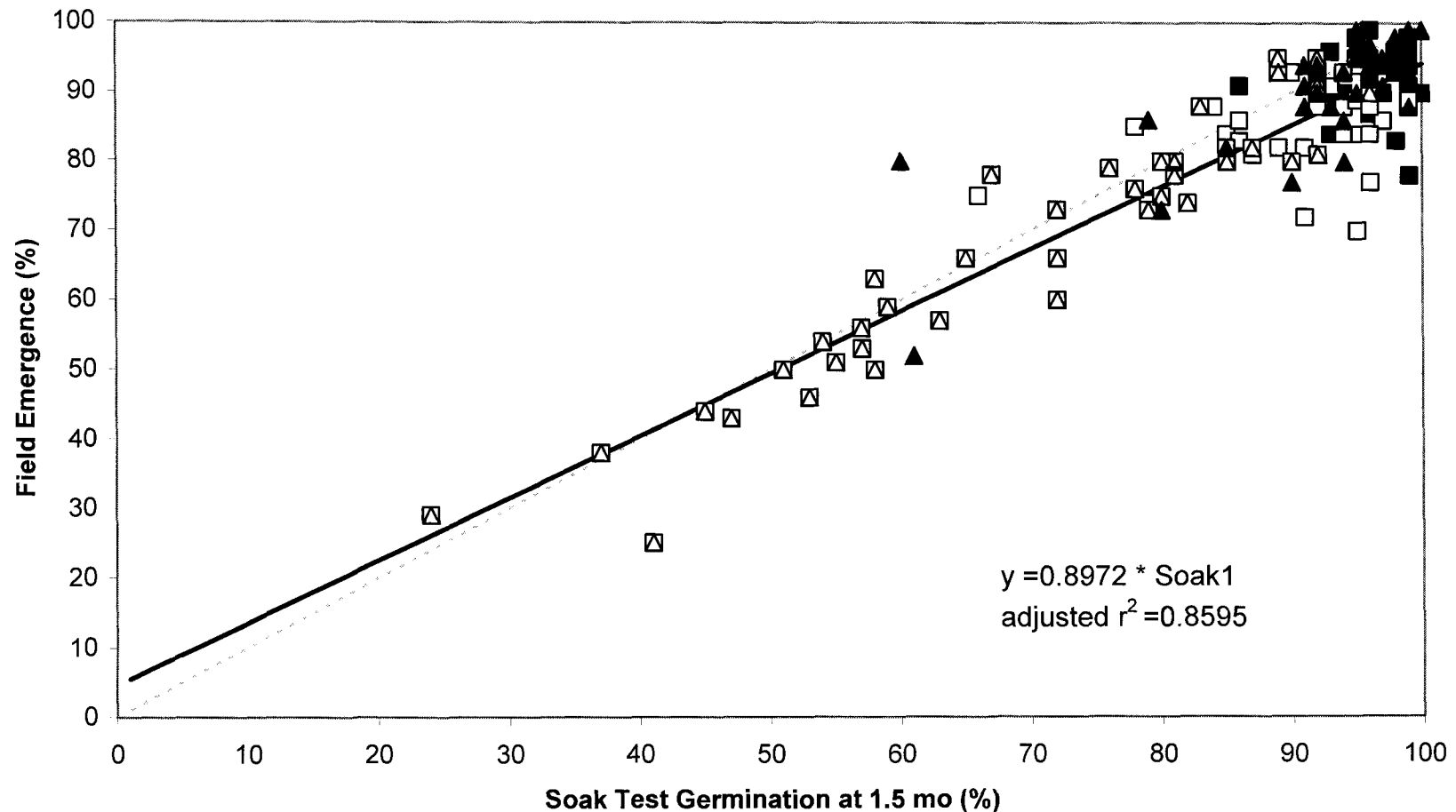
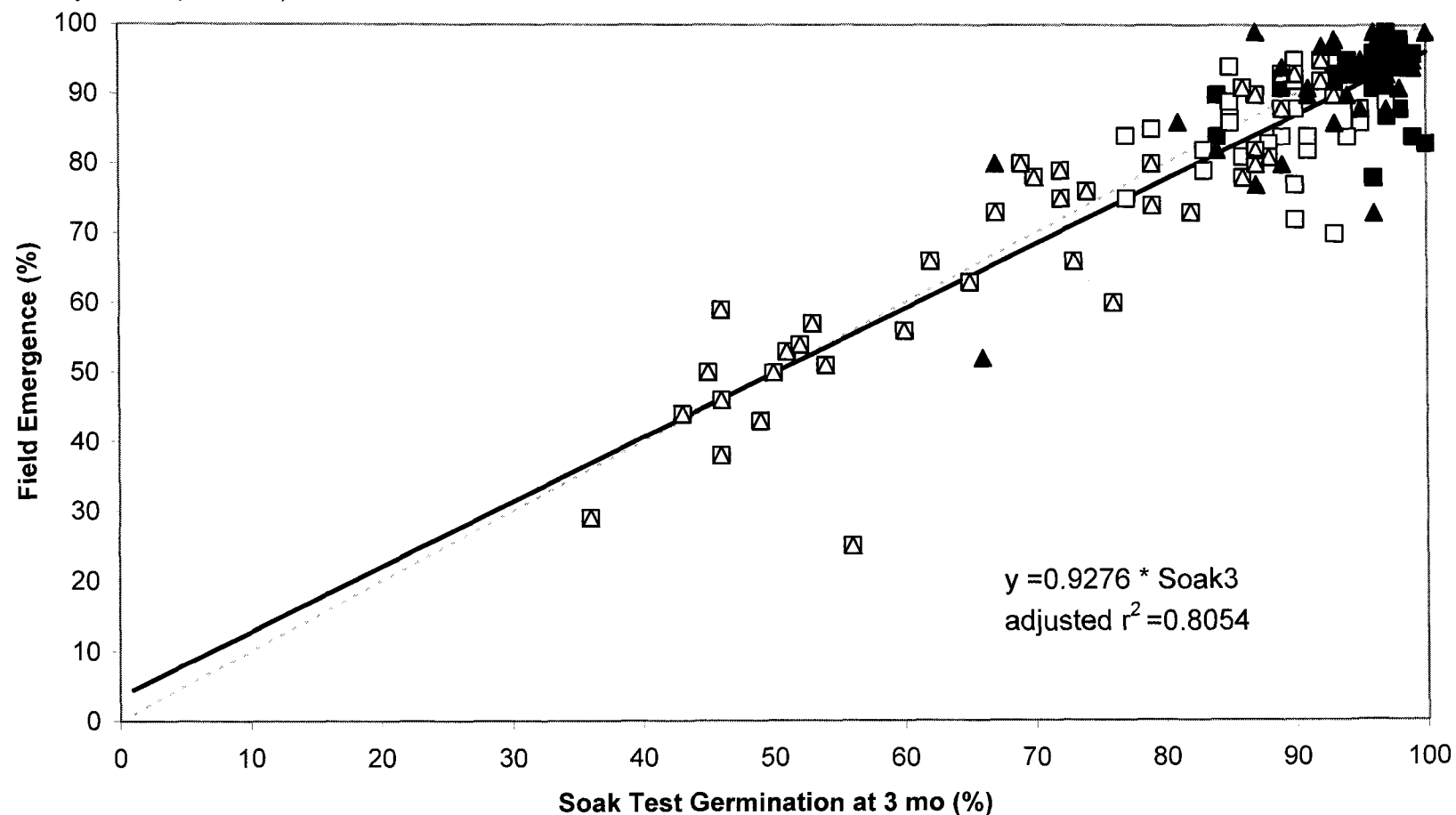


Figure 13. Soak test at 3 mo (Soak3) and late field emergence. Solid squares (■) represent 2003 non-frosted seed; open squares (□) represent 2003 frosted seed; solid triangles (▲) represent 2004 non-frosted seed; open triangles (△) represent 2004 frosted seed. Only frosted seed lots are in the regression, non-frosted samples are plotted for comparison. Dashed line is a 1x:1y ratio which represents the ideal slope for the regression between Soak3 and field emergence; solid line is the regression equation with significant factors. Some points overlap and therefore, both symbols (△ and □) are visible



## **CHAPTER 4. MEASURING EXPRESSION OF mRNA SEGMENTS IN DRY MAIZE SEED**

### **Introduction**

For many decades our understanding of abiotic stress in seeds was limited to the observation of physiological characteristics. Gene identification and sequencing ushered a new era of molecular research. Today, genes from stressed plants can be isolated and categorized. Technology has allowed scientists to move beyond gene identification into studying the function of the discovered genes. The development of tools for genetic profiling has facilitated this process.

Genetic profiling often refers to the use of microarrays followed by polymerase chain reaction (PCR). Microarrays are a key component for determining gene expression on a large scale. Thousands of genes can be screened using this technique. After this preliminary screening, a few promising genes can be further analyzed using reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR can be used to quantify expression of a sequence in a sample and compare this level of expression to other samples.

Genetic profiling has been used in a variety of applications. Zinselmeier et al. (2002) used gene expression profiling to explore the effects of shade and water stress on female reproductive tissue of maize. Their research not only provided new information on putative gene function, but also supported previous physiological research.

Frost damage in maize seed at the molecular level is not well understood. Frost events can lead to various physiological changes in developing and maturing seeds. Moisture content and genotype are two of the most important factors in determining the amount of damage due to frost (DeVries, unpublished). Freezing immature maize seed causes dehydration stress (Hartwigsen, 1999). Tolerance to freezing increases once acquisition of desiccation tolerance is reached. As seed matures and moisture content decreases, the impact of frost or freezing also decreases. Genotype also influences the response to premature dehydration and

frost (Rossman, 1949). Hartwigsen (1999) observed a change in expression of certain genes in seedlings grown from freeze-damaged seeds. These damaged seeds were obtained by artificially freezing the whole ears of corn prior to physiological maturity. This response indicates that seeds can activate alternative pathways during development in response to frost. In most cases the response to frost is multigenic (Thomashow, 1999). The author hypothesized that there is not one gene or gene family responsible for conferring frost tolerance. Instead, there are several families of genes with varied functions acting individually and collectively to increase tolerance to frost.

Some of the important gene families involved in seed development and maturation encode for products including: dehydrins (a class of late embryogenesis abundant (LEA) proteins), cold-responsive genes (*COR*), C-repeat/dehydration responsive element binding factors (*DREB/CBF*), heat-shock proteins (*HSP*), as well as several unclassified sequences. Dehydrins are common in many cereal species and are associated with dehydration (Close et al., 1989; Close and Chandler, 1990). Close (1996, 1997) indicates that dehydrins are found in many cellular compartments and therefore speculated that dehydrins may have several roles in cryoprotection. Dehydrins also have conserved regions which form amphiphilic  $\alpha$  helices (Dure et al., 1989). Although overexpressing a single dehydrin does not increase frost tolerance, co-overexpression of multiple dehydrins in *Arabidopsis* increases freezing tolerance (Puhakainen et al., 2004). The authors reported that the role of dehydrins is associated with membrane stabilization although the exact mechanism is still unclear. Increasing *COR* gene expression also increased *Arabidopsis* freezing tolerance (Jaglo-Ottosen et al., 1998). *COR* polypeptides are hydrophilic and homologous to group II LEA proteins (Thomashow, 1998). Steponkus et al. (1998) proposed that the *COR15am* polypeptide from the *COR15a* gene of *Arabidopsis* protected the chloroplast membrane by altering the curvature of the membrane. Artus et al. (1996) showed that *COR15a* expression also increased the stability of the entire protoplast by limiting interactions between the plasma membrane and the chloroplasts. *DREB/CBF*'s are transcription factors responsible

for altering gene expression in response to cold stress. Overexpression of *DREB1/CBF* increased chilling tolerance, but overexpression of *DREB2* did not (Nakashima and Yamaguchi-Shinozaki, 2006). *DREB1b/CBF1* is a transcriptional activator of the *COR* genes. The overexpression of *DREB1b/CBF1* enhances freezing tolerance beyond the tolerance achieved by overexpression a single *COR* gene (Jaglo-Ottosen et al., 1998). In a later study, Jaglo-Ottosen et al. (2001) found *CBF*-like genes in several flowering species, even in species that are cold-sensitive and non-acclimating like tomato.

One DREB in maize (*ZmDREB1a*) is induced by cold stress and is functionally similar to *DREB1/CBF* in *Arabidopsis* (Qin et al., 2004). In this study, overexpression of *ZmDREB1a* in *Arabidopsis* increased tolerance to freezing. Desikan et al. (2001) found that genes encoding for heat shock proteins were induced by hydrogen peroxide. These results indicate that oxidative stresses, found during premature dehydration or freezing, may increase *HSP* activity. *HSP101* is most abundant in the reproductive tissue and may be important in both low and high temperature stress (Young et al., 2001). Short heat shock proteins (sHSP's), like *CsHSP17.5* found in chestnut, may also play a role in the acquisition of freezing tolerance (Lopez-Matas et al., 2004). Lopez-Matas et al. (2004) found that *CaHSP17.5* is upregulated quickly in response to cold temperatures and acts as an effective cryoprotectant. All of these families of genes and their expressed proteins are important to understanding the response of plants to freezing temperatures.

There are two main types of gene expression, constitutive and inducible. Constitutively expressed regions are found in the entire plant all the time. These are often considered housekeeping genes. For example, Alcohol Dehydrogenase (*Adh1*) is considered constitutively expressed in maize. Actin and 18S are other examples of housekeeping genes. Induced expression can be spatial or temporal. Genes are expressed only for short periods or only expressed in certain tissues. The genes may be turned on and off by the plant itself. Other genes express based on an outside source either abiotic or biotic signal. *COR*, *RAB*, *HSP*, *LEA*, and several other families can all be stress induced (Cattivelli and Bartels, 1992).

Studies on the effect of stress focus on inducible expression. These sequences are then often overexpressed to determine if this confers tolerance to the stress.

While these gene families have been studied in-depth, there are several other sequences not attributed to a particular protein, but with high homology to sequences encoding for known proteins. For example, Hartwigsen and Goggi (2002) identified 15 sequences which were differentially expressed in maize seedlings grown from freeze-damaged seed. Three of these sequences of interest have high homology to proteins involved in metabolism including S-adenosylmethionine decarboxylase, Cytochrome P40 Monooxygenase, and Methionine Synthase (Hartwigsen and Goggi, 2002). The effect of frost on expression of these sequences in the seed has not been explored.

Unlike previous studies, we will extract RNA from the dry seed to see if expression or differential expression can be observed in the seed. The objective is to compare the expression of certain genetic sequences in frosted and non-frosted corn seed lots.

## **Materials and Methods**

### **Seed Production**

Twelve seed lots representing two genotypes harvested at three moisture contents were used in this study. Hybrids B73xIRF311 and Mo17xIRF311 were produced in isolation near Ames, IA. These two female lines were chosen to represent different genotypic tolerance to cold temperatures. B73 was developed by the federal-university corn breeding program at Iowa State University and is considered relatively tolerant to cold temperatures. Mo17 was developed by the federal corn breeding program at University of Missouri and is considered relatively sensitive to cold temperatures. IRF311 pollinator from the Iowa Research Foundation was selected based on flowering-synchrony with B73 and Mo17. Plants were harvested at 500-550, 400-450, and 300-350 g water kg<sup>-1</sup> fresh weight (fw). These moisture contents (MC) were selected to represent a range of maturity from middle of seed fill, immediately before physiological maturity, and directly after

physiological maturity, respectively. Ears were harvested with husk and shank intact. All ears were placed in 10°C for a minimum of 2 hours. Control samples were husked and dried in an experimental, forced air dryer until the seed was dry (140-150 g kg<sup>-1</sup>); while the remaining ears were moved to a Conviron growth chamber programmed with a 24 hour freeze cycle for the frost treatment. The frost cycle was developed to mimic typical early fall frost temperature patterns in Iowa (Dr. Elwin Taylor, personal communication). The entire cycle lasted 24 hours, beginning and ending at 10°C. It included 9 hours below 0°C and 2 hours at -5°C, which was severe enough to be considered a hard killing frost. After the freezing cycle was complete the ears were husked and dried in the experimental, forced air dryer until the seed was dry (140-150 g kg<sup>-1</sup>). Cordova-Tellez and Burris (2002) showed that drying corn seed with 400-450 g kg<sup>-1</sup> at 35°C did not cause damage and in fact mimicked maturation on the parent plant. Ears were shelled and the seed stored at 10°C and 50% relative humidity until RNA extraction.

#### RNA extraction

One hundred seeds were taken from each sample and ground in a blender (Waring Laboratory and Science, Torrington, Connecticut) according to the Iowa State Seed Laboratory experimental protocol until a uniform corn flour was obtained. Once the corn flour was homogeneous, approximately 100 µg were used for RNA extraction. Total RNA was extracted from the seed powder using TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA) according to the specified protocol. An additional isolation step suggested by Invitrogen was added prior to phase separation to reduce proteins, polysaccharides and extracellular material. The homogenate was centrifuged at 12,000 xg for 10 minutes at 4 °C. The supernatant was transferred to a fresh tube and phase separation proceeded. Extracted RNA was purified using PureLink<sup>™</sup> Micro-to Midi<sup>™</sup> RNA Total RNA Purification System (Invitrogen, Carlsbad, CA). Purified RNA was resuspended in DEPC treated water and quantified using a ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).



### Primer and Probe Design

The endogenous control for this study was the 18S small unit ribosomal RNA gene (Genbank accession number AF168884), which was used in previous studies in corn (Shou, 2003). The same primer and probe sequences were ordered and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The primer pair and probe for the gene of interest (Genbank accession number BQ740237), which encodes for S-Adenosylmethionine Decarboxylase, were designed using PrimerQuest (IDT, Coralville, IA). The best set was chosen based on  $T_m$  values (probe  $T_m$  at least 2 °C above primer  $T_m$ ) and location within the gene of interest. The sequences selected were forward primer AAGGCGGGAGTGCTTGATGAGTAA; reverse primer GCAACGTCGGAAGTTCACATGCTT; and probe CGTCGCAATTCGTGGTTGTCGTTTGGT. The target probe and endogenous control probe were labeled with FAM as the reporter dye on the 5' end and with BlackHole Quencher-1 at the 3' end.

### Quantitative qRT-PCR

Two-step PCR was selected for this study. Synthesis of cDNA was done using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA). Samples were not normalized before quantification step because this is unnecessary for relative quantification (RQ) analysis (ABI, 2005). TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used for the PCR amplification and detection step. PCR reactions were carried out in a total volume of 25 µl containing approximately 1 ng cDNA and 0.2 µM concentrations of the forward primer, reverse primer, and probe. Plate design is shown in Figure 1 with three reps per sample of both the endogenous control and gene of interest. Standard 96-well plates were placed in the ABI 7900HT Fast Real-Time PCR (Applied Biosystems, Foster City, CA) machine set for 35 cycles. Each cycle included melting for 15 s at 95 °C and annealing/extending for 1 min at 60 °C (ABI, 2005). The post-PCR products were electrophoresed on a 3% agarose gel containing ethidium bromide to confirm the amplicon size.

## Results and Discussion

Total RNA extraction and purification resulted in pure RNA from dry maize seeds. These results were unique because in the past, RNA was only obtained from seedling tissue grown from seeds. Improved RNA extraction kits allowed the successful separation of pure RNA from polysaccharides and cell wall components found in corn flour. Table 1 lists the approximate quantity and quality of RNA extracted from the seed samples as measured with the spectrophotometer. Ideal  $A_{260}/A_{280}$  readings for mRNA are around 2.0 (NanoDrop, 2006). The samples in this study had readings within this range. PCR products were run on a 3% agarose gel. Figure 2 shows the 110 bp amplicon for the 18S primers. The negative control (NTC) did not contain the amplicon. Figure 2 also shows the 174 bp amplicon for the gene of interest. The NTC did not contain the amplicon. Sample 4 did not contain either amplicon (lanes 5 and 19). Although pure RNA was obtained there was a problem with transcription during reverse transcriptase PCR and cDNA was not synthesized.

Previous PCR runs showed that after cycle 35 the FAM label started to break down, which led to false amplification signal in the NTC samples. Figure 3 is an example of the FAM and ROX signal for Sample 1 during a PCR run. As expected, the FAM signal amplified at the end when sufficient template was available and increased exponentially with each cycle. However, Figure 4 shows a NTC sample during a PCR run. The increase in FAM signal at the end of the run was due to probe breakdown and not amplification of DNA. This increase in FAM signal due to probe breakdown was not exponential. Randhawa et al. (2001) saw similar results with FAM and therefore only recommends using samples with a cycle threshold (CT) less than 38.

A standard curve was determined for both 18S endogenous control and the gene of interest for all samples. Standard curves for both the 18S and gene of interest were determined using a 10 fold (10 to  $10^8$ ) and 4 fold (4 to  $4^5$ ) dilution series, respectively. The quantity of cDNA in each reaction in the dilution series, for two representative samples (A and B), is shown in Table 2. Amplification plots and

standard curves for the 18S endogenous control and gene of interest, on one example sample each, are shown in Figures 5-8. The CT values of 18S and the gene of interest are evenly spaced across the dilution series in Figure 5 and 7, respectively. This indicates that the genes used in this study have a large dynamic range. The no template control (NTC) amplifies at the same time as the  $10^8$  dilution (Figure 5). This could be contamination or probe degradation. Running the PCR products on a 3% agarose indicated contamination because there was an amplicon the same size as the dilution series (data not shown). To corroborate this assumption, subsequent runs were made with a new order of primers and probe for 18S. As suspected, there was a contamination problem in the original run. The contamination was eliminated by using the newly synthesized primers and probes for 18S and discarding the original set. The  $r^2$  for the 18S standard curve for sample A was 0.988 (Figure 6). The  $r^2$  for the gene of interest standard curve for sample B was 0.997 (Figure 8). An  $r^2$  value of at least 0.95 was preferred for proceeding with quantification. Using the slope of the regression, the efficiency of the PCR reaction can be calculated (Ginzinger, 2002). The efficiency of the 18S PCR run was 85% and 108% for the gene of interest PCR run. These results indicated that the PCR reactions were successful and samples could be analyzed using absolute quantification or relative quantification.

These standard curves can be used for absolute quantification of the samples. However, the relative quantification curves were used in this study to show the effect of frost treatment. The amplification plot for the endogenous control and gene of interest for all twelve samples is shown in Figure 9. The 18S endogenous control amplified earlier than the gene of interest, indicating that it was more abundant and was more expressed. All samples amplified in a tight group for both genes. The NTC samples for both genes did not amplify, however some probe degradation was still seen in the 18S NTC at cycle 34. The  $\Delta\Delta CT$  values for each female and MC combination are shown in Table 3. The comparison between frost treatment and control for the Mo17 hybrid harvested at  $500\text{-}550\text{ g kg}^{-1}$  could not be made because of the transcription problem with sample 4.

The artificial frost treatment did not affect the expression of SAM decarboxylase in desiccated maize seeds. In Figures 10 and 11 the expression of SAM decarboxylase for all samples is compared to the expression of non-frosted seed at physiological maturity (300-350 g kg<sup>-1</sup>). This allows comparisons between the females and moisture contents. All samples normalized to Sample 6, the hybrid with Mo17 as the female parent harvested at physiological maturity and not frosted, are shown in Figure 10. The expression of the Mo17 hybrid did not change based on moisture content at harvest. However, all the B73 hybrid samples had lower levels of expression of the gene of interest than Sample 6. Figure 11 shows all samples normalized to Sample 12, the hybrid with B73 as the female parent harvested at physiological maturity and not frosted. This graph also shows that the Mo17 hybrid had a higher level of expression of the gene of interest compared to the B73 hybrid. The B73 hybrid harvested at three moisture contents did not vary in expression of the gene of interest. The samples from the Mo17 hybrid harvested at 300-350 g kg<sup>-1</sup> (Samples 5 and 6) and 400-450 g kg<sup>-1</sup> (Samples 7 and 8) had similar expression of the gene of interest. The Mo17 hybrid harvested at 500-550 g kg<sup>-1</sup> could not be compared because sample 4 did not contain cDNA.

There was no effect of frost on the expression of the gene of interest (Genebank accession number BQ740237) in desiccated maize seed. This indicates that S-Adenosylmethionine Decarboxylase (SAM decarboxylase) is not differentially expressed in response to frost during seed maturation. However, the two hybrids differ in their levels of expression of SAM decarboxylase. The Mo17 hybrid has significantly higher amounts of the SAM decarboxylase mRNA relative to the B73 hybrid. SAM decarboxylase converts SAM for Spermidine or Spermine synthesis. SAM decarboxylase is the rate limiting step for the polyamine biosynthetic pathway (Buchanan et al., 2000). Based on our findings, SAM decarboxylase transcription is not highly active in desiccated maize regardless of frost treatment.

## **Conclusions**

It is possible to extract mRNA from corn flour by using the latest kits available. The high quality and abundant quantity of mRNA obtained is adequate for PCR reactions. Relative gene expression can be measured using Real-Time PCR.

Although, the hypothesis was that the gene of interest was differentially expressed in maize seeds, our results do not support this statement. The expression of the gene of interest is very stable in maize seeds harvested at various moisture contents. We found that genotype is a more important factor than moisture content and frost treatment in determining expression of the gene of interest.

SAM decarboxylase is not differentially expressed in maize seeds in response to frost, but further work should be completed to examine the expression of other important gene families of maize. Using this procedure many other genes can be screened to determine the influence of frost on gene expression and metabolism. Because the maternal parent of the hybrid may be more significant than the environment (frost in this case), multiple genotypes should be used when screening other genes for effects of abiotic stress such as frost.

## **Acknowledgements**

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Table 1. Spectrophotometer readings of mRNA quality and quantity. Female B is B73 and M is Mo17; Moisture 50 is 500-550 g kg<sup>-1</sup>, 40 is 400-450 g kg<sup>-1</sup>, and 30 is 300-350 g kg<sup>-1</sup>; Treatment T is artificial frost, and C is control.

Sample	Block	Female	Moisture	Treatment	A260/A280	ng/ $\mu$ l
1	1	B	50	T	1.97	71.19
2	1	B	50	C	2.03	71.6
3	1	M	50	T	2.05	82.63
4	1	M	50	C	2.09	74.73
5	1	M	30	T	1.95	52.60
6	1	M	30	C	2.01	47.68
7	1	M	40	T	2.11	45.51
8	1	M	40	C	2.09	49.20
9	1	B	40	T	2.09	50.58
10	1	B	40	C	2.09	56.11
11	1	B	30	T	2.09	36.56
12	1	B	30	C	2.01	24.32

Table 2. Quantities of cDNA in PCR reactions for determining the standard curve for the 18S endogenous control and the gene of interest.

18S Dilution	cDNA from Sample A (ng)	Gene of Interest Dilution	cDNA from Sample B (ng)
$10^1$	1.23	$4^1$	4.66875
$10^2$	0.123	$4^2$	1.167185
$10^3$	0.0123	$4^3$	0.291796875
$10^4$	0.00123	$4^4$	0.072949219
$10^5$	0.000123	$4^5$	0.018237305
$10^6$	0.0000123		
$10^7$	0.00000123		
$10^8$	0.000000123		

Table 3.  $\Delta\Delta CT$  values for the gene of interest by female and MC combination.

	$\Delta CT^\dagger$	$\Delta CT$ SD	$\Delta\Delta CT$ (C-T)
Sample 1	15.782523	0.13574894	-0.307536
Sample 2	15.474987	0.09524518	
Sample 3	14.29689	0.15474804	
Sample 4			
Sample 5	15.139057	0.19289659	-0.1007147
Sample 6	14.774073	0.09218194	
Sample 7	14.807905	0.22538957	-0.1887423
Sample 8	14.711697	0.03664726	
Sample 9	15.445144	0.13940568	0.0548788
Sample 10	15.810883	0.08452687	
Sample 11	15.737765	0.19728947	-0.0767717
Sample 12	15.806405	0.12051778	

$^\dagger$   $\Delta CT$  values normalized to the 18S endogenous control CT values.

Figure 1. Plate design for Real-Time PCR of Block 1. GI master mix contains primers and probe for the gene of interest; 18S master mix contains primers and probe for the endogenous control; Blank column contained nothing and was not recorded; NTC (no template control) contains specified master mix and water.

Sample†	Master Mix							
	GI-1	18S-1	GI-2	18S-2	GI-3	18S-3	Blank	NTC
	#1	#1	#1	#1	#1	#1	Blank	18S Master
	#2	#2	#2	#2	#2	#2	Blank	18S Master
	#3	#3	#3	#3	#3	#3	Blank	18S Master
	#4	#4	#4	#4	#4	#4	Blank	18S Master
	#5	#5	#5	#5	#5	#5	Blank	18S Master
	#6	#6	#6	#6	#6	#6	Blank	18S Master
	#7	#7	#7	#7	#7	#7	Blank	Bob Master
	#8	#8	#8	#8	#8	#8	Blank	Bob Master
	#9	#9	#9	#9	#9	#9	Blank	Bob Master
	#10	#10	#10	#10	#10	#10	Blank	Bob Master
	#11	#11	#11	#11	#11	#11	Blank	Bob Master
	#12	#12	#12	#12	#12	#12	Blank	Bob Master

† Samples are listed in Table 1.

Figure 2. PCR product from all samples on 3% agarose gel. Lanes 1, 15, and 29 are 1 kb DNA ladder. Lanes 2-13 are PCR products of all samples (1-12, respectively) from 18S primers. Lane 14 is NTC for 18S. Lanes 16-27 are PCR products of all samples (1-12, respectively) from gene of interest primers. Lane 27 is NTC for gene of interest.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

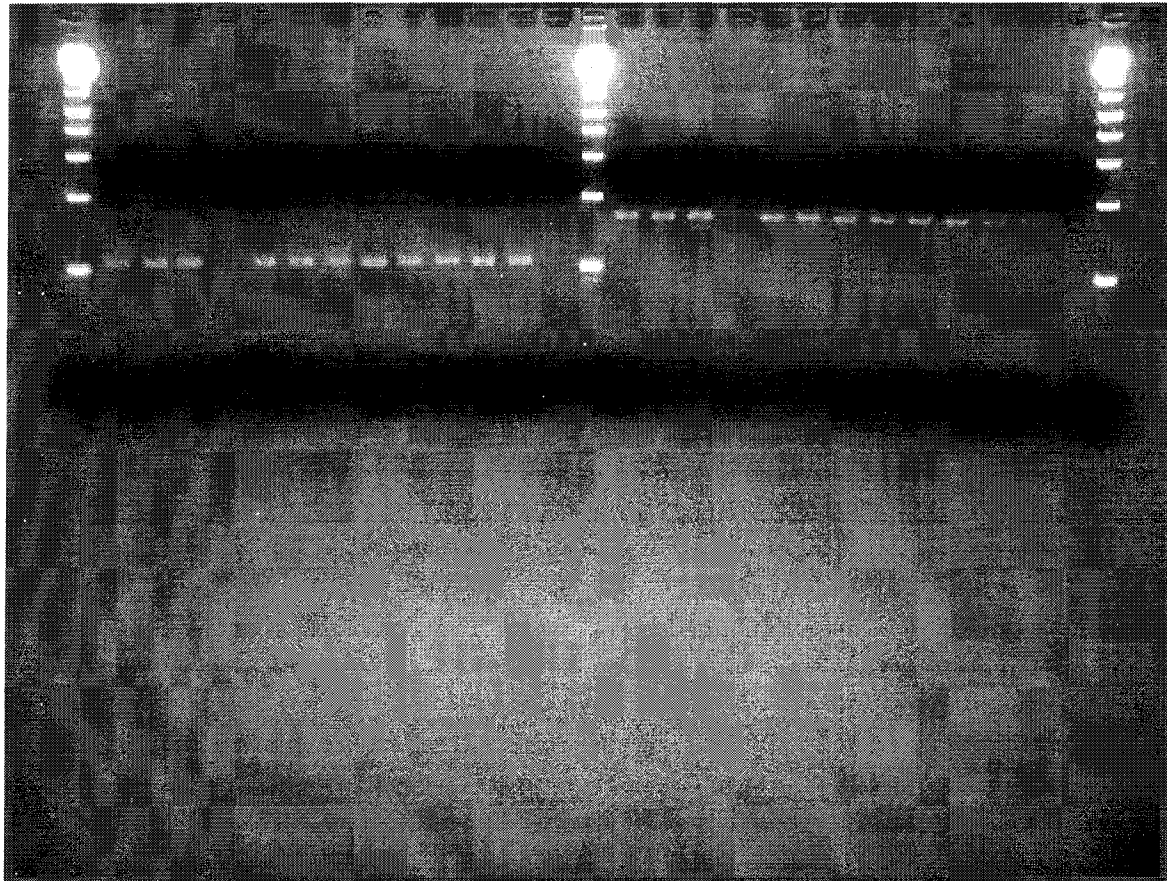


Figure 3. FAM and ROX background for Sample 1 with good amplification.

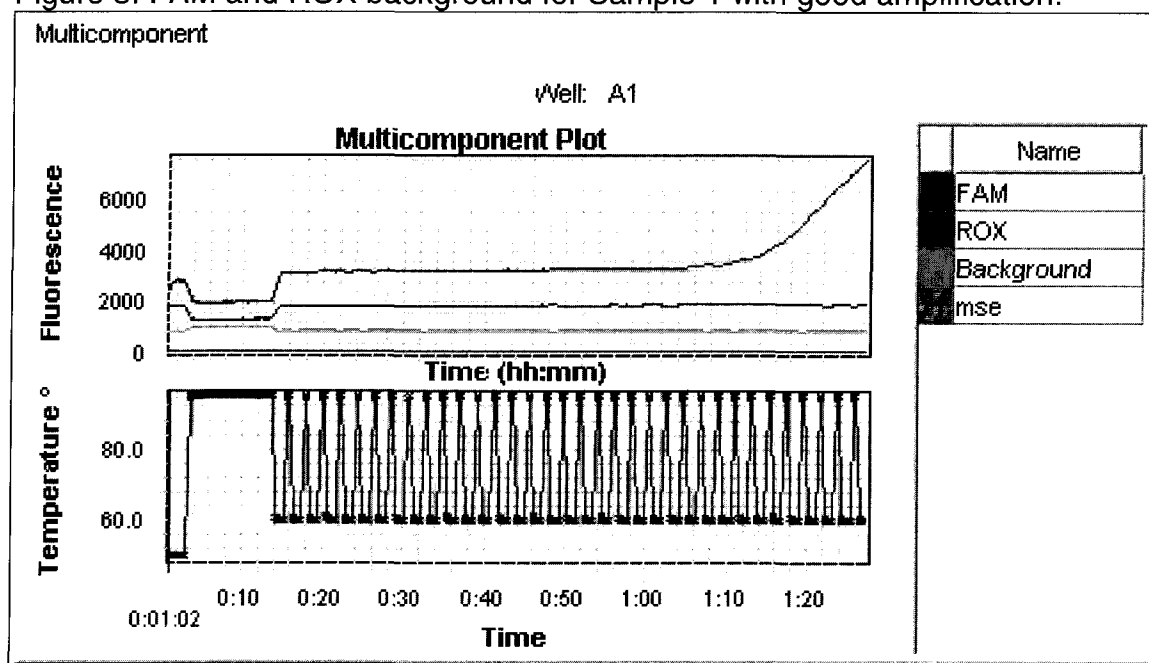


Figure 4. FAM and ROX background for a NTC sample showing FAM breakdown.

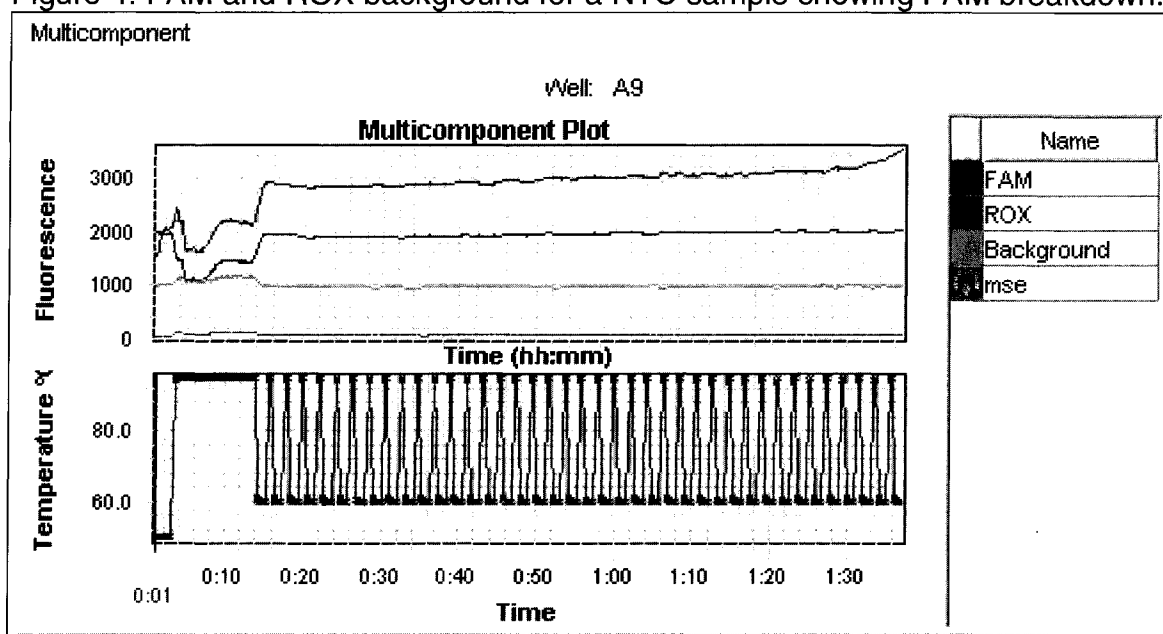




Figure 5. Amplification plot for 18S endogenous control gene in Sample A with 10 fold serial dilution (10 to 10<sup>8</sup>). No template control (NTC) shows base level of contamination as it crossed the threshold at the same point as the 10<sup>8</sup> dilution. Fluorescence threshold was set at 30. CT values are given at the top of the graph in red.

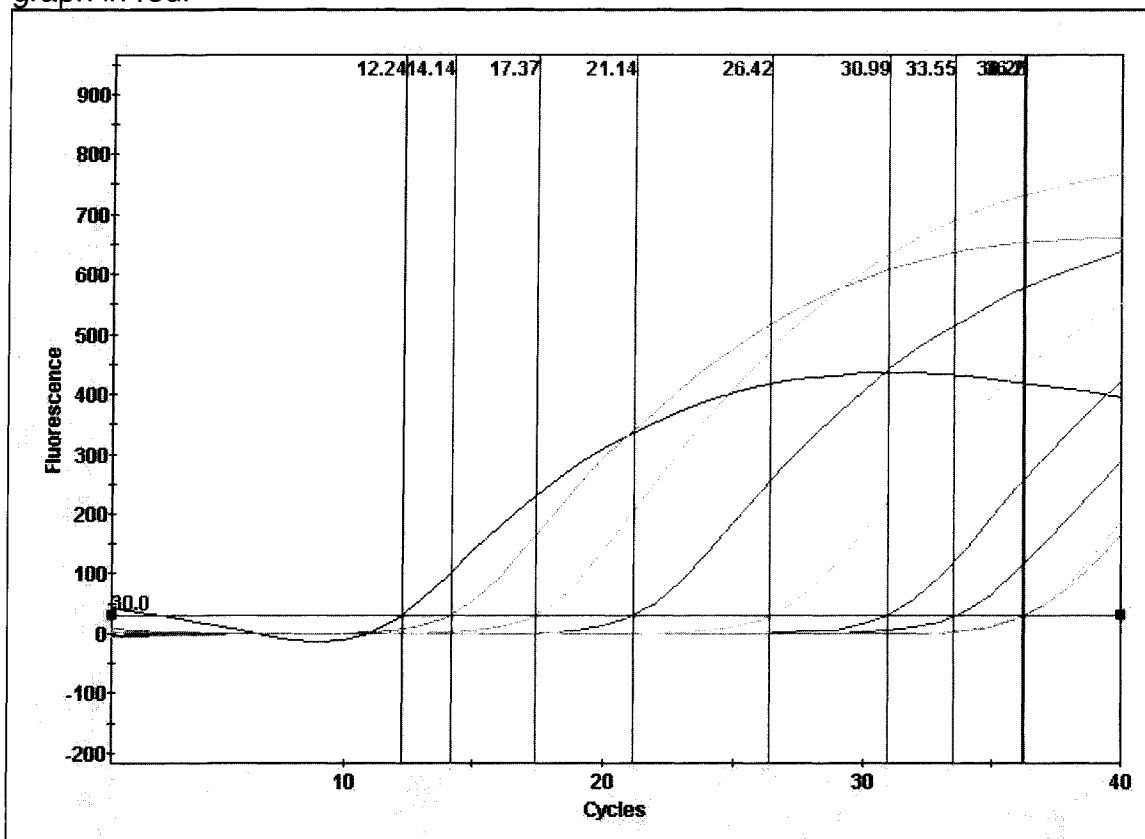


Figure 6. Standard curve for 18S endogenous control gene in Sample A with 10 fold serial dilution ( $10$  to  $10^8$ ).

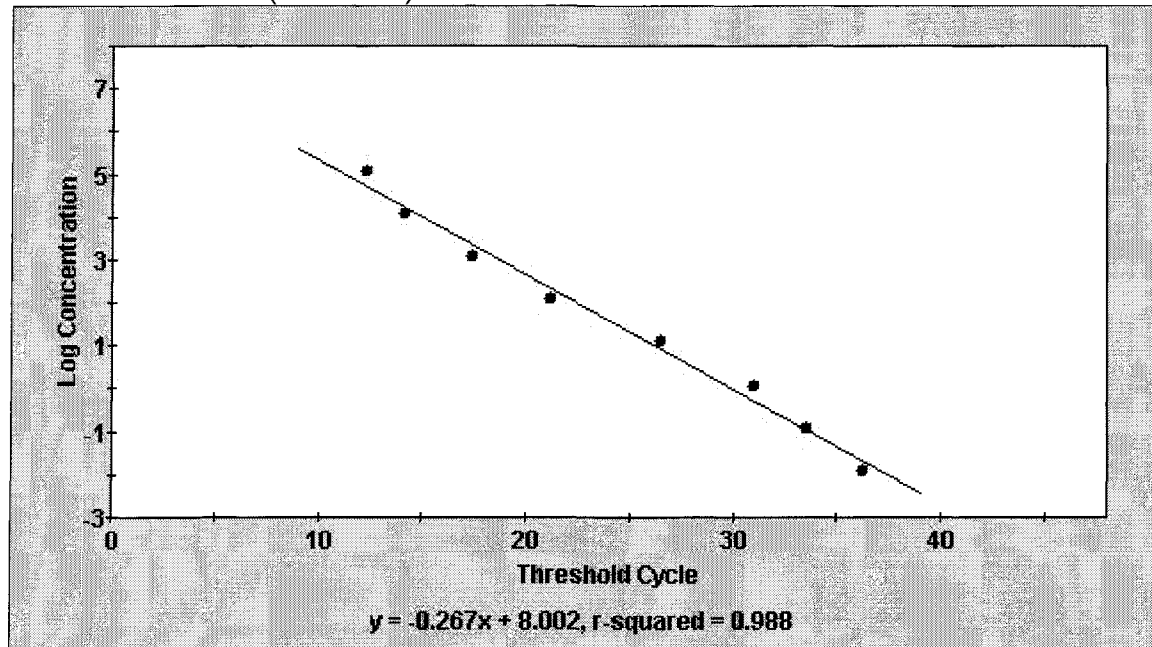


Figure 7. Amplification plot for the gene of interest (Genebank accession number BQ740237) in sample B with 4 fold serial dilution (4 to 4<sup>5</sup>). No template control (NTC) shows no amplification. Fluorescence threshold was set at 30. CT values are given at the top of the graph in red.

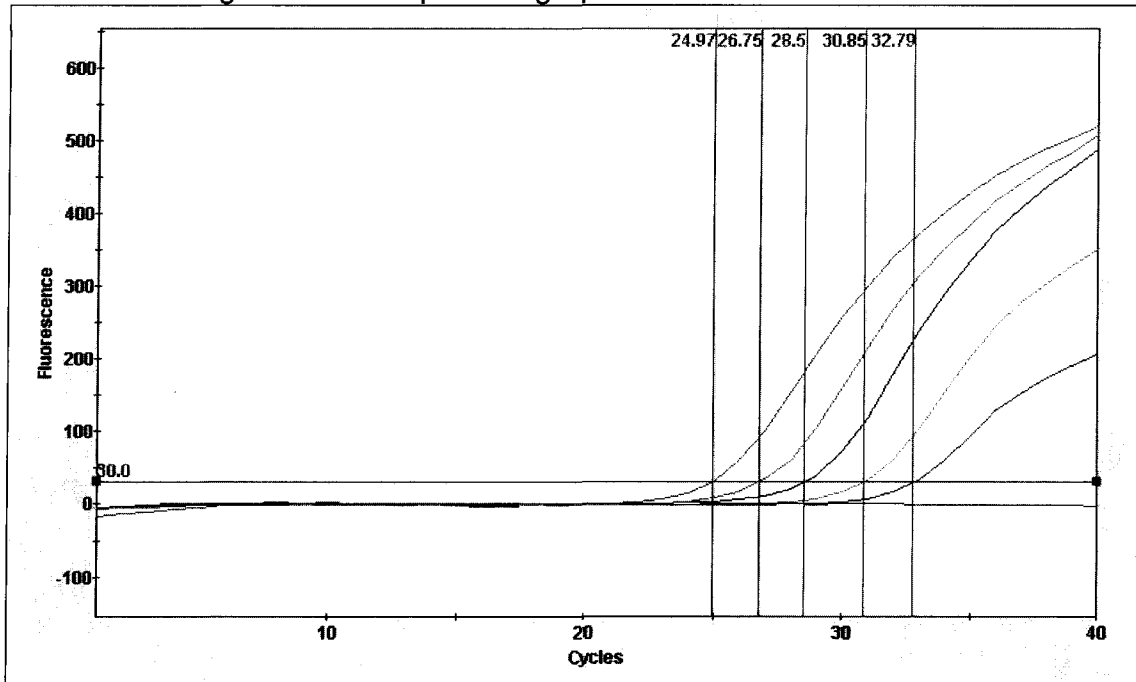


Figure 8. Standard curve for the gene of interest (Genebank accession number BQ740237) in Sample B with 4 fold serial dilution ( $4$  to  $4^5$ ).

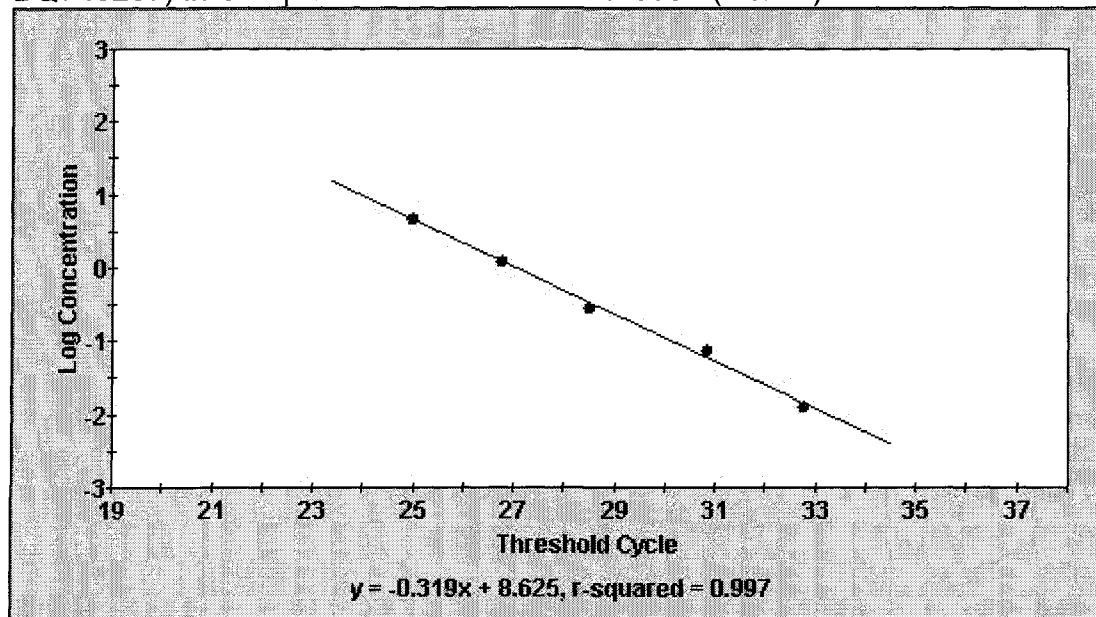


Figure 9. Amplification plot for all twelve samples including 3 replications of 18S and the gene of interest. Both genes are amplifying in very tight groups, and 18S is more abundant than the gene of interest.

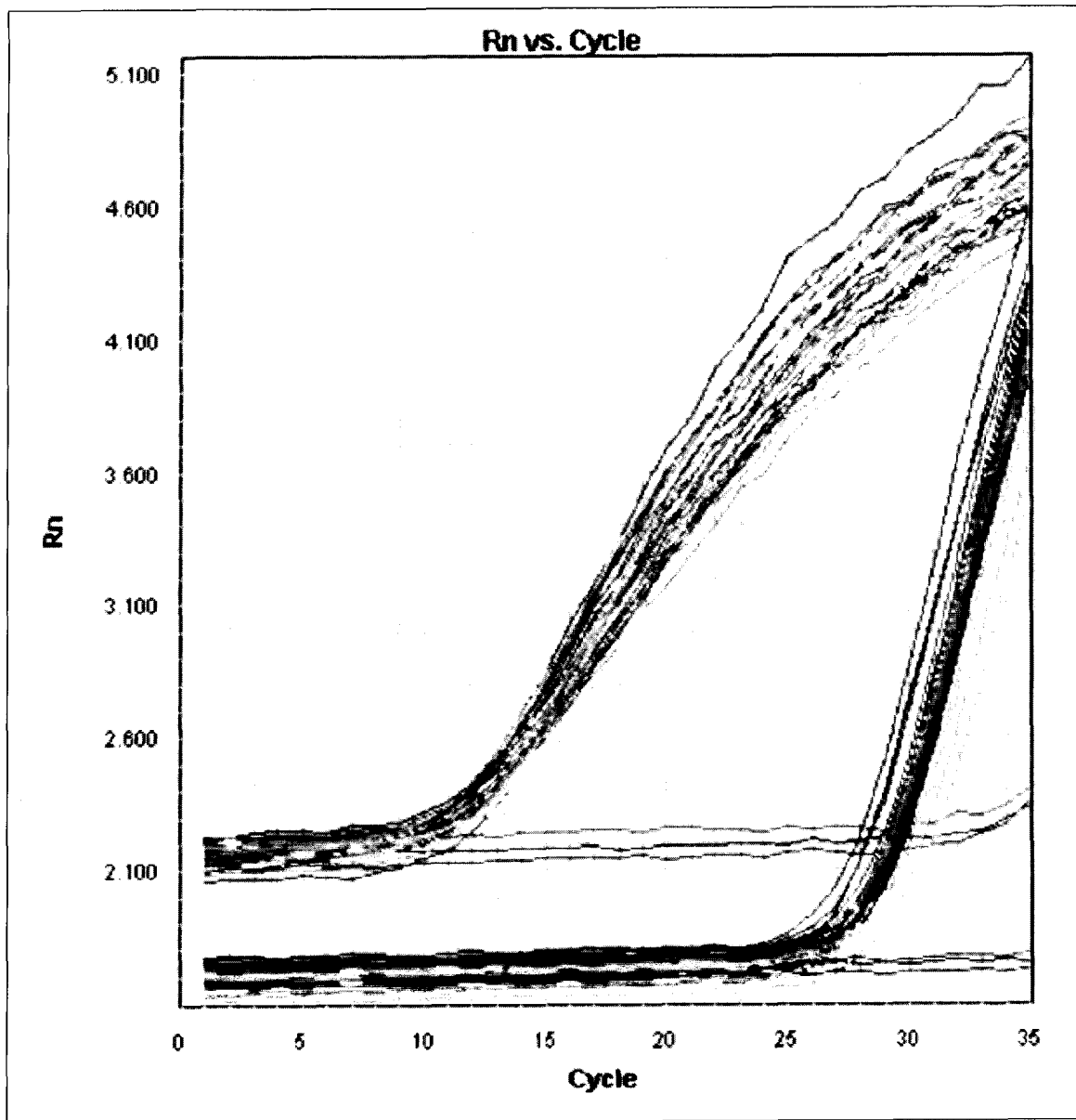


Figure 10. Relative expression of SAM decarboxylase with Sample 6 (Mo17 hybrid, harvested at 300-350 g kg<sup>-1</sup>, control) as the calibrator. Samples are listed in Table 1.

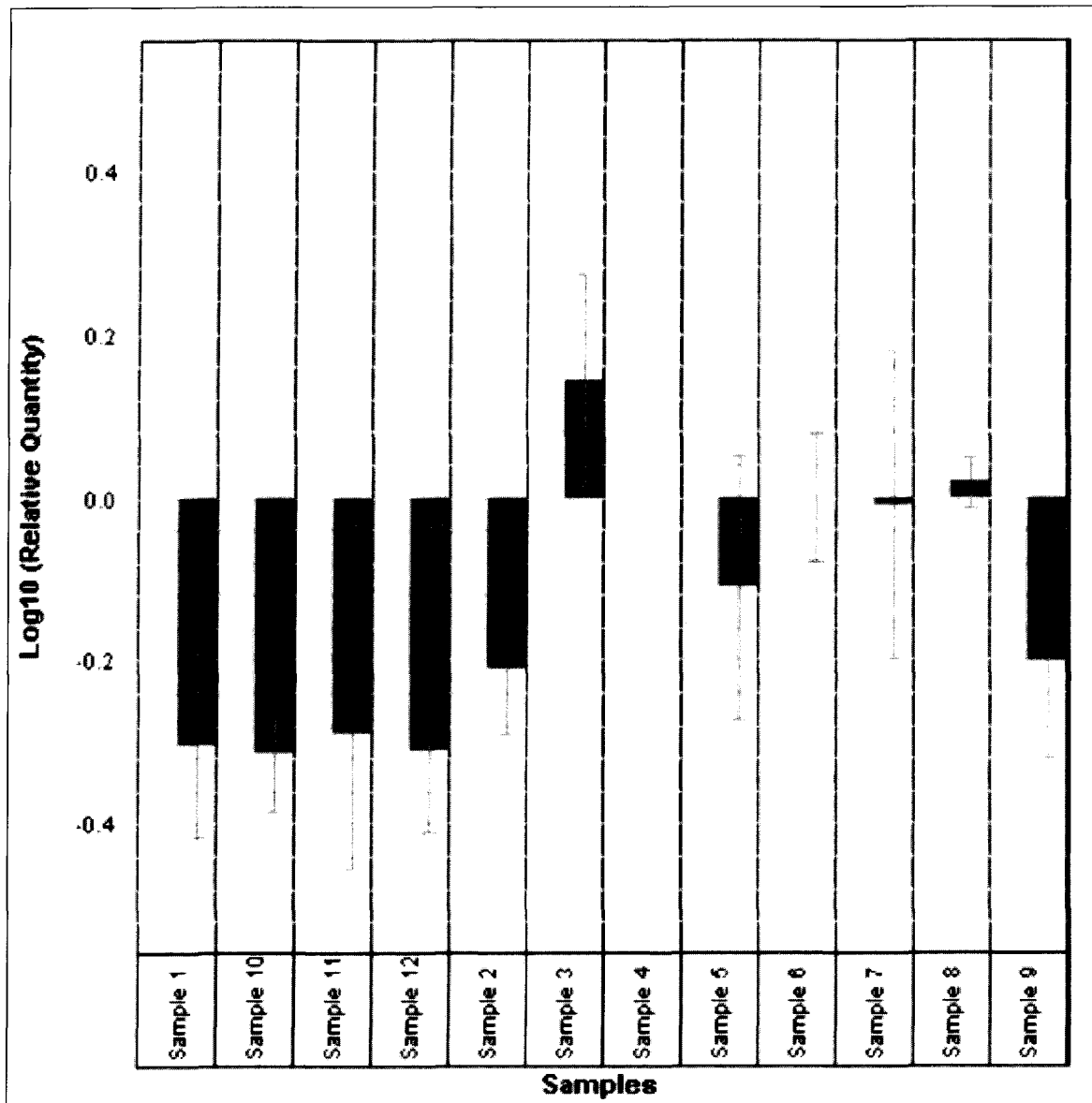
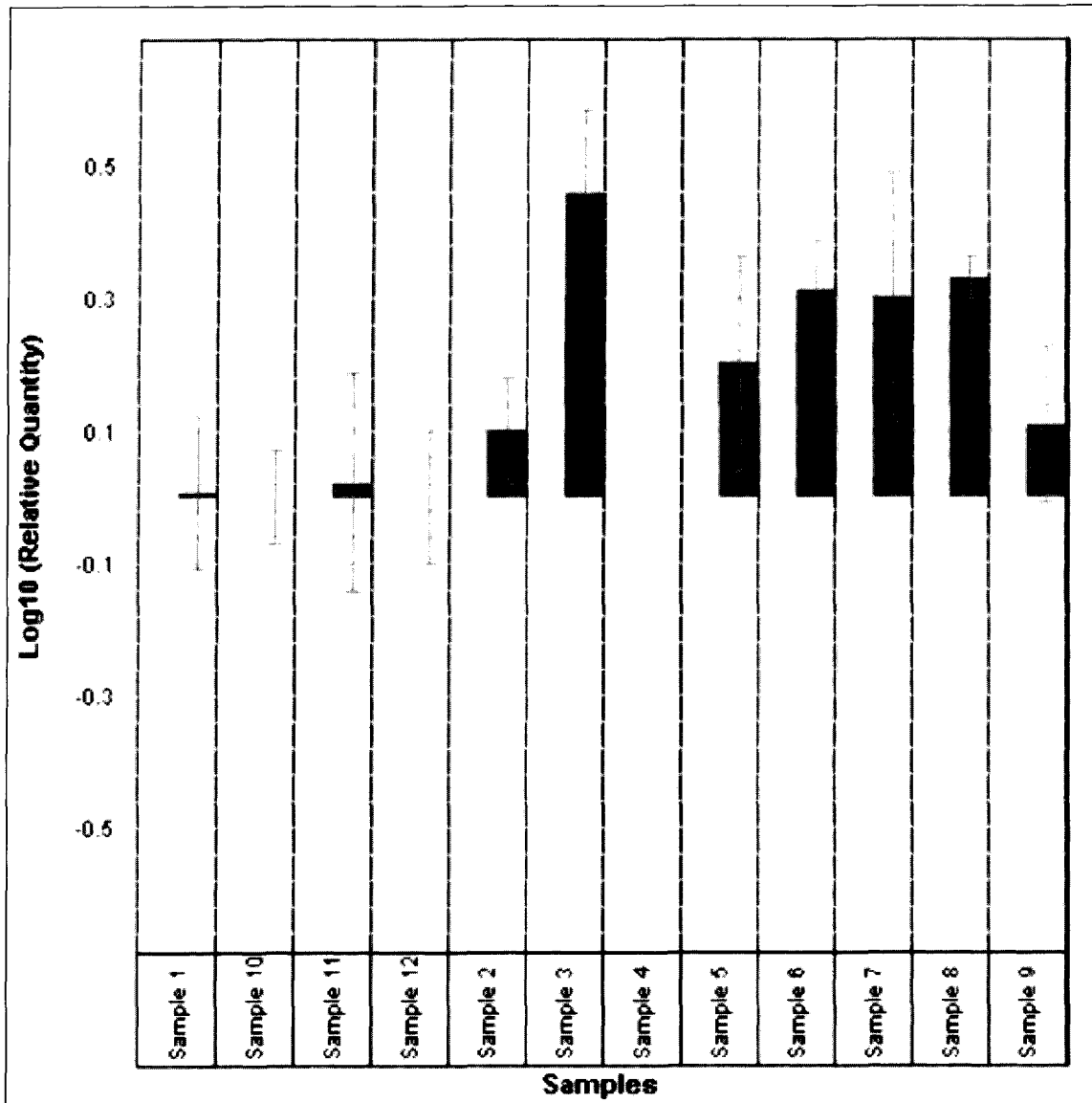


Figure 11. Relative expression of SAM decarboxylase with Sample 12 (B73 hybrid, harvested at 300-350 g kg<sup>-1</sup>, control) as the calibrator. Samples are listed in Table 1.



## CHAPTER 5. GENERAL CONCLUSIONS

Fall frost damage is a major threat to maize seed production in the central United States. Frost events prior to harvest can cause various physical, mechanical, biochemical, and physiological changes to immature seed corn. These changes can lead to decreased germination and vigor. Early detection of frost damage can reduce the financial loss caused by poor emergence when these seed lots are planted.

Many different aspects of frost damage have been explored in this project, which provides several methods for identifying frost damage in maize seed. This project advances our understanding of seed physiology as related to frost damage and changes in physiology during seed maturation.

The tetrazolium (TZ) test is still a very good diagnostic tool for identifying frost damage in maize seed. The new TZ staining procedure facilitates the evaluation and interpretation of staining patterns in frost-damaged seeds. Classifying viable seeds into separate vigor classes can be beneficial for research purposes or as a diagnostic tool, but it is not necessary for obtaining good estimates of germination or vigor after 6 months of storage.

The ability of common seed quality tests to predict field emergence of frost damaged seed varies. Standard germination and accelerated aging tests have strong relationships to field emergence in average to good field conditions. However, both tests overestimate field emergence. The saturated cold and soak tests are the best predictors of field emergence and seed quality of frost damaged seed lots. The results from these two tests are consistent during the first 3 mo of storage, and they are equal to each other in their ability to predict field emergence. We conclude that the tetrazolium, saturated cold, and soak tests are good seed quality tests for assessing frost damage and predicting field emergence of frosted seed lots.

Results from these experiments advance our understanding of the importance of seed moisture content and maternal parent on the extent of frost damage in immature maize seeds. Our study confirms previous reports that frost damage is



less severe as seeds mature and dry down. The hybrids produced from B73 and Mo17 as maternal parents respond differently to frost at the reproductive stages. The Mo17 hybrid was tolerant to frost at physiological maturity, while the B73 hybrid suffered significant decreases in viability and vigor.

A novel method for screening genes for differential expression in desiccated maize seed is presented. It is possible to extract mRNA from corn flour by using the latest RNA extraction kits. The high quality and abundant quantity of mRNA obtained is adequate for PCR reactions. Relative gene expression can be measured using Real-Time PCR. Although the original hypothesis was that the SAM decarboxylase is differentially expressed in maize seeds, our results do not confirm this hypothesis. The expression of the gene of interest is very stable in maize seeds. However, SAM decarboxylase levels in dry seeds do not change with the incidence of frost at the different levels of seed maturity tested. The genotype is a more important factor than moisture content and frost treatment in determining expression of the gene of interest. Further work should be done to examine the expression of other important gene families in maize seeds in response to frost.

The threat of an early frost event will always be a factor in seed corn production. However, the ability to estimate the impact of frost provides a scientific basis for management decisions in the event that a fall frost event occurs. This research also enhances our ability to identify and predict the field emergence of frost damaged maize seed using tetrazolium, saturated cold, and soak tests. Perhaps, based on the protocol suggested by this research, a molecular test capable of assessing frost damage can be developed in the future.

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